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PART III & IV

Arbuscular mycorrhizal research in coastal sand dunes : A review

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Abstract

Coastal sand dunes are extreme habitats with special reference to salinity, drought and disturbance. A variety of plant species and microorganisms have been adapted to such ecosystem. Arbuscular mycorrhizal (AM) fungi are known for their wide distribution in coastal sand dunes throughout the world. Besides facilitating the uptake of nutrient by plants, AM fungi play a vital role in succession of dune vegetation and formation of stable sand aggregates. Majority of the studies on coastal sand dune AM fungi and their application in sand dune restoration have been confined to temperate regions. Inventory and AM fungal application in tropical coastal sand dune restoration and stabilization are yet to be intensified. The present review has focused on : geographical distribution, species richness, diversity, dormancy and survival of AM fungi in coastal sand dunes; impact of sand dune edaphic features and disturbance on AM fungal activities; role of AM fungi in dune plant succession, dune restoration and stabilization; pattern of formation of wind-resistant sand aggregates and application of AM fungi in sand dune stabilization. The importance and future outlook of AM fungi in coastal sand dunes is also discussed.

(**Key words** : dune vegetation/succession/rhizosphere/sand aggregation/dune disturbance/edaphic features/arbuscular mycorrhizae/geographical diversity/species richness/endophytes).

Introduction

Coastal sand dunes are the low nutrient and unstable habitats of vital economic and ecological importance^{1,2}. Despite the geographical differences, coastal sand dunes have been considered as a specific ecosystem due to several common environmental features. The major limiting factors leading to the loss and instability of coastal sand dune ecosystem are : shoreline regression, salt spray, sandy texture and abrasion, sand accretion and erosion, low moisture and nutrient availability, high soil temperatures, herbivory and human interference³. Arbuscular mycorrhizal (AM) fungal associations have a wide range of habitats : aquatic, deserts, coastal sand dunes, tropical rain forests and canopy epiphytes⁴. Although the research efforts from 1970 onwards concentrated mainly on the occurrence of AM fungi in field conditions⁵, knowledge on functioning of AM fungi in the field is limited^{6,7}. In field studies the main focus was on agricultural ecosystems, whereas the natural ecosystems and disturbed ecosystems have been neglected⁸. The AM fungi are widely distributed biota in coastal sand dunes throughout the world⁹. They play an important role in plant succession and stabilization of maritime sand dunes by facilitating nutrient uptake, binding sand grains into stable aggregates and improving soil structure¹⁰.

The present review addresses the role of AM fungi in coastal sand dunes. The major subtopics considered for discussion include : inventory in different continents; species richness and diversity; seasonal periodicity; sand aggregation and dune stability; role in the establishment and succession of maritime vegetation; spore dormancy and germination; spore ageing, parasitism and death; effect of edaphic factors; impact of disturbance; effect of endophytes; AM fungal status of non-mycorrhizal and facultatively mycorrhizal plant species.

Inventory

Inventory is the first step to understand the role of microorganisms in an ecosystem. Coastal sand dunes are favourable for the establishment and functioning of AM fungi mainly because of their low phosphorus content^{2,11}. Ever since the importance of AM fungi in sand dune succession has been recognized¹², many surveys have been conducted in temperate, subtropical and tropical coastal sand dunes of the world.

Temperate locations :

Inventory of AM fungi in coastal sand dunes of temperate locations were mainly confined to the United States and Europe. More attention has been focused on the

Atlantic coast of United States. In Rhode Island, *Ammophila breviligulata* (American beach grass) dominates the dune vegetation and exhibits greatest spore density in the rhizosphere¹¹. Studies on the spore population of five species of AM fungi in coastal sand dunes of Massachusetts revealed the antagonistic interactions between them¹³. Twenty three species of AM fungi were recovered from the major plant species growing on barrier dunes along 355 km coastal stretch extending from northern New Jersey (*A. breviligulata*) to Virginia (*A. breviligulata*, *Myrica pensylvanica*, *Solidago sempervirens* and *Uniola paniculata*)¹⁴. Studies on six locations along the transect showed an increase in overall mean species richness of AM fungi in all plant species from north to south. Several new species of AM fungi have been described from the sand dunes of temperate locations of the Atlantic and Pacific coasts (Table 1). *Gigaspora gigantea* is the most dominant AM fungal species in the sand dunes of Atlantic coast of United States¹⁵.

Table 1.— New species of arbuscular mycorrhizal fungi described from the maritime sand dunes.

Taxon	Location	Reference
Acaulosporaceae		
<i>Acaulospora elegans</i> Trappe & Gerd.	Orgeon, USA	110
<i>A. gadanskensis</i> Balszkowski	Chalupy, Poland	111
<i>A. koskei</i> Balszkowski	Slowinski, Poland	112
<i>A. polonica</i> Balszkowski	Hel, Poland	111
<i>Entrophospora baltica</i> Balszkowski, Madej & Tadych	Northwest, Poland	113
Gigasporaceae		
<i>Scutellospora arenicola</i> Koske & Halvorson	California, USA	114
<i>S. coralloidea</i> (Trappe, Gerd. & Ho) Walker & Sanders	Oregon, USA	110
<i>S. dipapillosa</i> (Walker & Koske) Walker & Sanders	Rhode Island, USA	115

Table 1, Contd...

<i>S. erythroa</i> (Koske & Walker) Walker & Sanders	Rhode Island, USA	21
<i>S. fulgida</i> Koske & Walker	Virginia, USA	116
<i>S. hawaiiensis</i> Koake & Gemma	Kaua'i, Hawaii	79
<i>S. pellucida</i> (Nicol. & Schenck) Walker & Sanders	Florida, USA	116
<i>S. persica</i> (Koske & Walker) Walker & Sanders	New Jersey, USA	115
<i>S. reticulata</i> (Koske, Miller & Walker) Walker & Sanders	Rhode Island, USA	117
<i>S. verrucosa</i> (Koske & Walker) Walker & Sanders	Virginia, USA	115
<i>S. weresubiae</i> Koske & Walker	Virginia, USA	116
<i>Glomaceae</i>		
<i>Glomus corymbiforme</i> Balszkowski	Swinoujscie, Poland	118
<i>G. gibbosum</i> Balszkowski	Swinoujscie, Poland	119
<i>G. globiferum</i> Koske & Walker	New Jersey, USA	120
<i>G. halon</i> Rose & Trappe	Lincolnshire, England	121
<i>G. microaggregatum</i> Koske, Gemma & Olexia	California, USA	122
<i>G. microcarpum</i> Tulasne & Tulasne	Idaho and California, USA	110
<i>G. minutum</i> , Balszkowski, Tadych & Madej	Northwest, Poland	123
<i>G. monosporum</i> Gerd. & Trappe	Oregon, USA	110
<i>G. nanolumen</i> Koske & Gemma	Kaua'i, Hawaii	124
<i>G. pallidum</i> Hall	Tautuku, New Zealand	125
<i>G. pansihalos</i> Berch & Koske	California, USA	126
<i>G. pustulatum</i> Koske, Friese, Walker & Dalpe	Rhode Island, USA	127
<i>G. trimurales</i> Koske & Halvorson	California, USA	114

Studies on European coastal sand dunes confined mainly to Italy and Poland. On the Italian dunes, members of the family Asteraceae, Papilionaceae and Poaceae were heavily colonized^{16,17}. Two plant species belonging to the family Caryophyllaceae were also colonized by AM fungi in stable sand dunes of Italy¹⁶. The well-fixed dunes at Migliarino, Italy showed varied AM fungal flora similar to that in the dunes of Australia¹⁸ and Rhode Island¹¹. A significant correlation was found between spore density and extent of root colonization. Maritime sand dunes of Poland yielded several new and interesting species of AM fungi (Table 1).

Subtropical locations :

Sand dunes of many subtropical locations were surveyed for the existence and activity of AM fungi. Such locations include : Florida, Mexico, Baja California, Brazil, Japan, Australia and Pakistan. Root samples of 37 plant species, along the succession gradient of mobile to stable dunes of the Gulf of Mexico revealed the colonization of 97% plant species by AM Fungi¹⁹. The mycorrhizal inoculum potential increased with dune stabilization. Plant species belonging to the so-called non-mycorrhizal families (*Aizoaceae* and *Cyperaceae*) were also colonized by AM fungi. Root colonization gets initiated during the rainy season and as the soil dries, arbuscules decline and vesicles begin to increase as observed in the dunes of Baja California²⁰. The phenology of plant species and fungal species coincides. *Scutellospora erythroa* described from Bahamas was common in sand dunes of North America²¹. *Acaulospora scrobiculata* was the most abundant species, while *Gigaspora albida* was the most frequent species in Brazilian dunes⁹.

In Japanese coastal sand dunes, Asai²² first encountered AM fungi colonization in dune plants. A total of six species of AM fungi was recorded from Japanese dunes²³. Among them *Glomus* spp. were most dominant. The highest density of spores was found to be associated with the rhizomes and the spores were detected up to a depth of 130 cm. Logan *et al.*²⁴ studied 41 plant species along the coast of New South Wales, Australia. Out of them 36 species were mycorrhizal. Interesting feature was that the members of the so-called non-mycorrhizal family (*Aizoaceae*, *Brassicaceae*, *Cyperaceae* and *Chenopodiaceae*) were also colonized by AM fungi. Koske¹⁸ studied the coastal sand dune vegetation at New South Wales at different stages of succession, which revealed the greater spore density in stabilized old dunes than in recent and mobile dunes. Among rest of the subtropical locations, coastal sand dunes of Pakistan revealed the occurrence of only one kind of AM fungus, its spore density ranging between 0.01 and 0.3/g²⁵.

Tropical locations :

Compared to temperate and subtropical locations, very few surveys are available on AM fungi of maritime sand dunes of tropical region. Relatively the sand dunes and vegetation of Hawaiian Islands have been investigated more intensively. Hawaii Islands got erupted from the sea nearly 30 million years ago. It was exposed to continuous volcanic activities and sand dune formation²⁶. These islands consist of many sand dune habitats, which are in the early stages of primary succession. The volcanic activities resulted in continuous selection of vegetation where mycotrophy played an important role. Most of the seedlings grown on the drift line area were associated with AM fungi²⁷. Roots of 23 out of 31 vascular plant species showed AM fungal colonization. Mycorrhizal index was highest in 12 plant species²⁷. Koske²⁸ screened eight sand dune plant species and recorded 12 species of AM fungi. *Sclerocystis sinuosa* and *Glomus microaggregatum* were dominant. A survey of sixteen plant species from the dunes of Kaua'i, Hawaii revealed the occurrence of 14 species of AM fungi²⁹. *Scutellospora hawaiiensis* was the common species. Dead and parasitized spores outnumbered the live AM spores.

A few surveys are available from the Indian subcontinent. Along the coast of Chennai covering 36 km transect, 56 plant species were surveyed by Mohankumar *et al.*³⁰. The rhizosphere of all plant species possessed AM fungi, but roots of only 35 plant species had AM fungal colonization. Among the members of the family Cyperaceae, *Cyperus stoloniferous* colonized by AM fungi, while *Kyllinga brevifolia* was not colonized. The rhizosphere of 12 plant species established on the west coast of India in post-monsoon season yielded the spores of 16 species of AM fungi³¹. Colonization of roots ranged from 34 to 80 percent. Spores of *Scutellospora gregaria* was most abundant, while *Glomus albidum* was most frequent. On non-vegetated dunes, spores of five species were recovered, among them *Glomus albidum* and *G. lacteum* were dominant. Two years seasonal survey of AM fungi associated with *Ipomoea pes-caprae* and *Launaea sarmentosa* on the sand dunes of west coast of Karnataka revealed the occurrence of 41 and 28 species respectively^{32,33}. Colonization of roots of AM fungi was at a peak during post-monsoon season, but the spore density was maximum during summer (*I. pes-caprae*) or monsoon (*L. sarmentosa*). The AM fungal phenology (colonization, spore density and species richness) was coincided with the plant phenology which might have increased the fitness of *I. pes-caprae* and *L. sarmentosa* on the coastal sand dunes. The diversity of AM fungi associated with *I. pes-caprae* on 10 sand dunes at a stretch of 100 km of west coast of India consist of moderately disturbed dunes (MDD) and severely disturbed dunes (SDD) were studied

during wet and dry seasons by Beena *et al.*³⁴. The vegetation cover, AM colonization, spore richness and diversity were greater in MDD than in SDD irrespective of the season. The AM fungal richness and spore density was strongly correlated with rhizosphere nitrogen. *Glomus mosseae*, *G. dimorphicum* and *Gigaspora gigantea* were most common in both MDD and SDD.

A survey conducted on the plant species of coastal land of Singapore after five years of reclamation did not show the presence of AM fungi³⁵. Non-mycorrhizal plant species were prevalent in reclaimed area. The success and persistence of non-mycorrhizal plant species on reclaimed area was due to the lack of AM propagules and competition from mycorrhizal plant species. A gradual increase in colonization of mycorrhizal plant species away from the reclaimed area was seen, the rhizosphere of *Casuarina equisetifolia* consists of *Gigaspora rosea* and *Rhynchelytrum repens* and *Desmodium heterophyllum* consist of *Glomus macrocarpum*.

Comparison of AM Flora

Distribution :

Comparison of AM flora on different sand dunes by Koske and Tews³⁶ revealed a maximum similarity between the Rhode Island and New Jersey to Virginia. Although the AM fungi have a broad host range, the observed differences in similarity might be due to the independent responses of beach grasses, AM fungi and a set of ecological factors³⁶. The AM fungal community of Hawaiian Islands is very distinct from that of Australia, Atlantic and Pacific coasts of United States, Scotland and Italy²⁸. So far about the 76 species of AM fungi have been recorded from the coastal sand dunes (Table 2). Relatively more studies have been conducted from the sand dunes of temperate regions than the tropical and subtropical regions. Due to this disparity, comparison of species richness and diversity between the wide geographical regions may not be relevant. From Table 2 it can be deduced that 49, 38 and 23 species have been reported from temperate, tropical and subtropical regions respectively. Out of the 76 species, 11 species (*Acaulospora scrobiculata*, *Gigaspora albida*, *G. gigantea*, *Scutellospora calospora*, *S. erythroa*, *S. gregaria*, *S. pellucida*, *Glomus aggregatum*, *G. constrictum*, *G. fasciculatum* and *G. globiferum*) are cosmopolitan in distribution. *Acaulospora scrobiculata* is frequent in south-eastern Australia, Brazil and Atlantic dunes of United States. *Gigaspora gigantea* and *Glomus aggregatum* are common in east coast of North America. *Scutellospora erythroa* is a common member of the sand dunes of Atlantic coast of the United States. *Glomus fasciculatum* was encountered by Kulkarni *et al.*³¹ in the west coast of India, it is also widely distributed

in California to British Columbia. *Gigaspora albida* and *Scutellospora erythropa* were more frequent and *Gigaspora globiferum* and *S. calospora* were frequent in the west coast of India³¹⁻³⁴

Table 2.—Geographical distribution of arbuscular mycorrhizal fungi on coastal sand dunes.

(*Temperate locations—(A) North America : 11, 13, 14, 21, 45, 68, 83, 110, 114, 122, 127, 128, 129. (B) Europe : 16, 17, 38, 110, 112, 113, 118, 119, 121, 123, 129. (C) Japan : 23, 41.

Subtropical locations—(D) Australia : 18, 125, 129, 130. (E) Florida : 40, 58, 128, 131. (F) Brazil : 9.

Tropical locations—(G) Hawaiian Islands : 28, 29, 79. (H) India : 30–32, 34. (I) Singapore : 35).

Taxon	Location*
Acaulosporaceae	
<i>Acaulospora elegans</i> Trappe & Gerd	A
<i>A. denticulata</i> Sieverding & Toro	H
<i>A. gadanskensis</i> Balszkowski	B
<i>A. koskei</i> Balszkowski	B
<i>A. lacunosa</i> Morton	A
<i>A. laevis</i> Gerd. & Troppe	A, B, D, E
<i>A. mellea</i> Spain & Schenck	A
<i>A. morrowiae</i> Spain & Schenck	E
<i>A. nicolsonii</i> Walker, Reed & Sander	H
<i>A. polonica</i> Balszkowski	B
<i>A. scrobiculata</i> Trappe	A, D-G
<i>A. spinosa</i> Walker & Trappe	H
<i>A. taiwanica</i> Hu	H
<i>Entrophospora baltica</i> Balszkowski, Madej & Tadych	B

Table 2, Contd...

<i>E. infrequens</i> (Hall) Ames & Schneider	A, H
<i>E. schenckii</i> Sieverding & Toro	H
Gigasporaceae	
<i>Gigaspora albida</i> Schenck & Smith	A, E, F, H
<i>G. decipens</i> Hall & Abbott	H
<i>G. gigantea</i> (Nicol. & Gerd.) Gerd. & Trappe	A, D, E, H
<i>G. gregaria</i> (Schenck & Nicolson) Walker & Sanders	H
<i>G. margarita</i> Becker & Hall	A, H
<i>G. rosea</i> Nicolson & Schenck	A, I
<i>Scutellospora arenicola</i> Koske & Halvorson	A, H
<i>S. calospora</i> (Nicol. & Gerd.) Walker & Sanders	A, B, D, H
<i>S. coralloidea</i> (Trappe, Gerd. & Ho) Walker & Sanders	A, F
<i>S. dipapillosa</i> (Walker & Koske) Walker & Sanders	A, E
<i>S. erythroa</i> (Koske & Walker) Walker & Sanders	A, E, H
<i>S. fulgida</i> Koske & Walker	A, E
<i>S. gilmorei</i> (Trappe & Gerd.) Walker & Sanders	A
<i>S. gregaria</i> (Schenck & Nicolson) Walker & Sanders	A, C, F-I
<i>S. hawaiiensis</i> Koske & Gemma	G
<i>S. nigra</i> (Redhead) Walker & Sanders	H
<i>S. pellucida</i> (Nicol. & Schenck) Walker & Sanders	A, E, H
<i>S. persica</i> (Koske & Walker) Walker & Sanders	A, B
<i>S. reticulata</i> (Koske, Miller & Walker) Walker & Sanders	A
<i>S. scutata</i> Walker & Diederichs	F

Table 2, Contd..

<i>S. verrucosa</i> (Koske & Walker) Walker & Sanders	A, E
<i>S. weresubiae</i> Koske & Walker	A, E, F
Glomaceae	
<i>Glomus aggregatum</i> schenck & Smith	A, C, E, G, H
<i>G. albidum</i> Walker & Rhodes	H
<i>G. caledonium</i> (Nicol. & Gerd.) Trappe & Gerd.	A
<i>G. claroides</i> Schenck & Smith	H
<i>G. clarum</i> Nicolson & Schenck	A, H
<i>G. constrictum</i> Trappe	A, F-H
<i>G. convolutum</i> Gerd. & Trappe	H
<i>G. corymbiforme</i> Balszkowski	B
<i>G. deserticola</i> Trappe, Blows & Menge	E, H
<i>G. dimorphicum</i> Boyetchko & Tewari	H
<i>G. etunicatum</i> Becker & Gerd.	A, F
<i>G. fasciculatum</i> (Thaxter) Gerd. & Trappe	A, B, D, H
<i>G. fecundisporum</i> Schenck & Smith	A, H
<i>G. fistulosum</i> Skou & Jakobsen	H
<i>G. gibbosum</i> Balszkowski	B
<i>G. globiferum</i> Koske & Walker	A, E, H
<i>G. halon</i> Rose & Trappe	B
<i>G. intraradices</i> Schenck & Smith	G, H
<i>G. lacteum</i> Rose & Trappe	H
<i>G. macrocarpum</i> Tulasne & Tulsane	I

Table 2, Contd..

<i>G. microaggregatum</i> Koske, Gemma & Olexia	A, G, H
<i>G. microcarpum</i> Tulasne & Tulasne	A, H
<i>G. minutum</i> Balszkowski, Tadych & Medaj	B
<i>G. monosporum</i> Gerd. & Trappe	A, H
<i>G. mosseae</i> (Nicol. & Gerd.) Gerd & Trappe	B, H
<i>G. nanolumen</i> Koske & Gemma	G
<i>G. occultum</i> Walker	A, H
<i>G. pallidum</i> Hall	D
<i>G. pansihalos</i> Berch & Koske	A
<i>G. pubescens</i> (Saccardo & Ellis) Trappe & Gerd.	A, H
<i>G. pustulatum</i> Koske, Friese, Walker & Dalpe	A, H
<i>G. reticulatum</i> Bhattacharjee & Mukerji	H
<i>G. spurcum</i> Walker	G
<i>G. tortuosum</i> Schenk & Smith	A, C, H
<i>G. trimurales</i> Koske & Halvorson	A
<i>Sclerocystis pachycaulis</i> Wu & Chen	H
<i>S. rubiformis</i> Gerd. & Trappe	A
<i>S. sinuosa</i> Gerd. & Bakshi	G

Spore density :

Mean or range of spore density of AM fungi in maritime sand dunes is given in Table 3. The spore density in Atlantic coast ranged from 0-3.4/g, while in Europe it was 0.05-0.95/g. Relatively the spore density in subtropical regions is higher than in temperate regions (0-11/g vs. 0-3.4/g). The spore density in tropical dunes ranged between 0 and 3.4/g.

Table 3.—Mean or range of spore density of arbuscular mycorrhizal fungi in coastal sand dunes.

Location	Spore density	Reference
Temperate locations		
Massachusetts, USA	0-0.7/g	85
Massachusetts, USA	0.24/g	128
Massachusetts, USA	0.32/ml (<i>Gigaspora gigantea</i>)	13
Rhode Island, USA	2.04	11
Rhode Island, USA	1.5-2.4/g	132
Rhode Island, USA	0.12-0.46 (<i>Gigaspora gigantea</i>)	39
Rhode Island, USA	1.92-3.36/g	47
New Jersey-Virginia, USA	0.64/ml	14
Alberese, Follonica and Migliarino, Italy	0.95/g (<i>Glomus</i> sp.)	16
Tuscany, Italy	0.05-0.95/g	17
Tentsmuir, Scotland	0.06-0.62/ml	34
Subtropical locations		
New South Wales, Australia	1-11/g	18
Hasaki, Japan	2.87/g	41
Shirako, Japan	6.56/g	41
Florida, USA	0-6.77/g	36
Florida, USA	0.42-2.4/g	60
Baja California, Mexico	0-8/g	20
Santa Catarina, Brazil	1.7-3.8/g	9

Table 3, Contd..

Pakistan	0.01-0.3/g	25
Tropical locations		
Singapore	0.15/g	35
Kaua'i, Hawaii	0.09/ml	29
Mangalore, India	0.75/g	31
Mangalore, India	0-3.4/g	32
Mangalore, India	0-2.1/g	33
West coast, India	0.05-0.44/g	34

New species :

About 29 species of AM fungi have been described from the maritime sand dunes (Table 1). Out of this, 25 species have been described from temperate locations alone, among the rest, two each have been described from subtropical and tropical locations. This clearly indicates the lack of intense studies on AM fungi in tropical and subtropical sand dunes.

Seasonal Periodicity

It is known that AM fungi sporulate differentially with change in time and host plant species³⁷. The AM species richness and spore abundance was significantly correlated with the age of plants. The dune vegetation in fixed coastal sand dunes of Scotland revealed two peaks of root colonization during July and October-November³⁸. A six-months seasonal study of AM fungal colonization of seven plant species in fixed coastal sand dunes of Baja California revealed up to 80% colonization²⁰. Arbuscules were more when the soil was moist and vesicles were more abundant in dry condition. Gemma and Koske³⁹ studied spore abundance of the dominant fungus *Gigaspora gigantea* in the dunes of Rhode Island. The spore abundance increased from July and reached a peak in December indicating that a majority of spores formed during late summer and fall. The abundance of healthy and dead spores was reported to be opposing in the dunes of Rhode Island¹⁵. Seasonal dynamics of spores of five AM fungi in a sand dune of Massachusetts showed a bimodal trend of peak of spore abundance during May and October¹³. The spore density was highest during the month of summer (August) than in spring (May) and

fall (November) in the dunes of Florida⁴⁰. Seasonal survey of AM fungi associated with *Spartina ciliata* in the dunes of Brazil did not show significant variations in spore abundance because of season⁹. But the spores of *Acaulospora* sp., *Glomus constrictum* and *G. etunicatum* were high in winter, while *Gigaspora albida* peaked during spring. No seasonal pattern was observed in AM spore number of three plant species in Japanese coastal sand dunes⁴¹. Several investigators observed the correspondence between the plant and fungal phenology^{32,33,42-45}. Similar observations were made in the sand dune vegetation of Baja California²⁰.

Species Richness and Diversity

The AM fungi in most rhizosphere systems indicate the local species composition, while it has a strong historical component with ecological process⁴⁶. In Australia the species richness was 1.5 in foredunes and it increased to 2.4 in the second dune ridge¹⁸. The mean species richness for the entire transect of New Jersey to Virginia was 4.9 (4.2-6.3) (ranged from 2-8 per root zone for *Ammophila* and *Solidago*, while for *Uniola* it was 6-14)¹⁴. The mean species richness was positively correlated with distance along transect and with temperature parameters. In Brazilian sand dunes the AM fungal richness index ranged from 4.6 (spring) to 6.8 (winter) with a mean of 5.9 for all samples⁹. The species richness of Brazilian sand dunes was similar to that of Atlantic coast of United States¹⁴, but it was higher than that of the southern Australia¹⁸ and Hawaiian Islands²⁸. The AM fungal richness and diversity was consistently greater in moderately disturbed dunes than in severely disturbed dunes³⁴. The spatial dispersion of AM species associated with the rhizosphere of American beach grass was analyzed at the depths of 10-18, 20-28 and 30-38 cm on the sand dunes of Rhode Island⁴⁷. A highest of 3.36/g of AM spores was recovered at 20-28 cm depth. The spore abundance was significantly correlated to the depth of 20-28 cm and 30-38 cm than 10-18 cm. In Japanese coastal sand dunes, in rhizosphere of *Elymus mollis* AM spores were present up to a depth of 90-130 cm⁴¹. Maximum spores were recovered at 30-60 cm depth.

Sand Aggregation

Mycorrhizae are known to improve the soil structure and stability due to the formation of sand aggregates⁴⁸. Three distinct phases of aggregate formation have been recognised : (i) AM hyphae entangle the soil particles; (ii) the roots and AM hyphae create conditions favourable to form microaggregates in soil; (iii) the roots and AM hyphae enmesh and bind the microaggregates to form larger macroaggregates⁴⁹. The AM fungal hyphae entangles microaggregates and the polysaccharide

secretion firmly adheres the microaggregates. Up to 50 m of fungal hyphae form a network per gram stable microaggregate⁵⁰. The AM fungal mycelia in sand dune soil stabilize the aggregates by the amorphous deposits of mycelia⁵¹. The AM fungi found in maritime sand aggregates along with bacteria, algae and other fungi⁵². A study of the association of *Glomus* with *Phaseolus vulgaris* showed that the weight of sand aggregates in non-mycorrhizal bean plants was 10g/kg, while it was 54g/kg in mycorrhizal plants⁵³. The weight and the size of the aggregates increase from mobile to fixed sand dunes. The amount of aggregations usually increases with distance from the sea due to decreasing salinity, elevated nutrients and establishment of higher plant species. The AM fungal association with mycorrhizal plant in the maritime sand dunes stabilizes the dunes by binding the sand grains into wind resistant aggregates. The aggregates of sand dunes remain intact even after the death of roots and hyphae¹⁰.

Association with Sand Dune Vegetation

In temperate locations most of the dune stabilizing plants belonging to the family Poaceae⁵⁴. American beach grass is a major dune building plant species adapted to north-eastern Atlantic coast of United States⁵⁵. This plant species has the greatest influence on coastal geomorphology and plant community development⁵⁶. Areas of the dunes with highest vegetation cover by American beach grass were associated with greatest AM spore density and diversity¹¹. The species richness of AM fungi was nonlinear in the rhizosphere of American beach grass and *Solidago* along the latitudinal temperature gradient¹⁴. This phenomenon has been attributed to two reasons : (i) an increase in richness due to the effect of increased temperature from north to south; (ii) stimulatory effects of host plants due to warmer temperature towards south. The decline in species richness along further south was expected due to increased heat stress on the hosts. *Solidago* is also less frequent in southern Atlantic coast. Most favourable conditions (particularly temperature) are found at mid zones between New Jersey to Virginia for plant species as well as AM fungi.

Under coastal sand dune stabilization programme, *Ammophila* has been planted extensively along mid and north Atlantic coast of United States⁵⁷. Artificial inoculation of AM fungi enhanced the transplanting success and establishment of *Ammophila*⁵⁶. Planting *Ammophila* during October to April is ideal to stabilize the sand dunes of New England coast. This period corresponds to ideal temperature and moisture levels for survival of beach grass⁵⁸. This period also corresponds to the peak of healthy inoculum of AM fungus, *Gigaspora gigantea*. Studies have been conducted on the pattern of AM fungal association of a large scale planting (age 0-7 years) of

Ammophila at Massachusetts. Over the successional sequence there was increase in the extent of AM fungal colonization of roots, community of AM spore population and the mycorrhizal inoculum potential of soil. The non-vegetated sites lack AM fungal propagules.

Ammophila arenaria (European beach grass or marram grass) is native to European coastal dune ecosystem. The AM fungal colonization was more extensive in the plant species established under European beach grass of British sand dunes⁵³. The AM fungal colonization was increased from foredunes to fixed dunes in Scotland⁵⁹. Inoculation of *Glomus macrocarpum* and *G. fasciculatum* stimulated the growth of European beach grass grown in non-stabilized foreshore of Scotland⁴⁰. South east Atlantic coast is naturally stabilized by perennial grasses particularly *Uniola paniculata* (sea oats)⁶⁰. Association of sea oats with AM fungi reduces its environmental stresses and facilitates a rapid growth and dune stability. The established sea oats in Florida beach replenishment site showed elevated AM fungal root colonization, cfu of AM fungi and cfu of bacteria than in vacant and recently planted beach sites⁶⁰. Performance of three AM isolates from Florida dunes (*Glomus deserticola*, *G. globiferum* and *G. etunicatum*) was tested on sea oats in green house trials⁶¹. Two isolates, *Glomus deserticola* and *G. etunicatum* significantly increased the height, dry mass and plant cover of shoots of sea oats compared to controls. *Glomus globiferum* increased the shoot phosphorus content significantly, but did not affect the plant growth. Seedlings of sea oats inoculated with *G. deserticola* and *G. macrocarpum* at Miami beach, showed greater shoot dry mass (219%), root length (81%), plant height (64%) and number of tillers (53%) compared to the uninoculated plants⁶². Bacterial inoculation (*Klebsiella pneumoniae* or *Alcaligenes denitrificans* with *Glomus* spp. to sea oats enhances the AM root colonization⁶³. Inoculation of *K. pneumoniae* increased the spore germination and hyphal growth of *G. deserticola*. Nitrogen fixing bacterial inoculation particularly *K. pneumoniae* enhances the AM symbiosis.

Most of the plant species established in the Gulf of Mexico (on the beach, embryo dunes, foredunes and stabilized dunes) were colonized by AM fungi¹⁹. In field bioassay, colonization of the seedlings of *Chamaecrista chamaecristoides* by AM fungi was more pronounced in the foredunes, but no mycorrhizal structures was noticed in mobile dunes¹⁹. Observation of seven dune plant species (from the beach, embryo dune, foredune and mobile dunes) suggested that the AM fungal colonization is crucial for the growth of early successional species⁶⁴. The fixed coastal sand dunes of Brazil have a high AM fungal diversity and it has been related to the dominance of rhizomatous perennial grass, *Spartina ciliata*⁹. Members of the family *Asteraceae*,

Convolvulaceae, *Fabaceae* and *Poaceae* are dominant in tropical coastal sand dunes^{51,65,66}. It was noticed that mycorrhizal indices were significantly higher in endemic plant species than in alien plant species at Hawaii Islands⁷⁷. High incidence of AM fungi has been found in the strand habitats of Galapagos Islands⁶⁷ and Heron Island, Great Barrier Reef⁶⁸. High incidence of AM fungi in Hawaii Island strand species agrees with the studies in Heron Island⁶⁸.

Spore Dormancy and Germination

The survival of AM fungal spores in sand dunes is dependent on biotic and abiotic factors. The spore dormancy facilitates overcoming the unfavourable conditions. The physicochemical features of coastal sand dunes influence the AM spore dormancy and germination. Different species of AM fungi may have different strategies of survival under wet and dry regimes of coastal sand dunes⁶⁹. Spores of *Gigaspora* sp. lose their appearance, colour and viability when stored for period longer than 6-8 months due to ageing⁷⁰. The dormancy of 2-9 weeks was seen in newly formed spores serving to maintain a high inoculum of AM fungi in soil during non-growing season of the host plant species *Ammophila* in Rhode Island³⁹. The spores of *Gigaspora gigantea* gathered from the rhizosphere of *Ammophila* in Rhode Island, germinated on sand immediately after collection at 2% moisture and at temperature as low as 20 °C⁷⁰. The spores of *G. gigantea* are not dormant in the absence of plant roots. Low temperature and low water potential prevent or delay the spore germination. Lack of such field condition in the sand dunes may not enforce spore dormancy. The AM fungi exhibits lower tolerance to higher carbon dioxide tensions and higher oxygen requirements for germination than other fungi⁷¹. The effects of sodium chloride on non-coastal AM fungal species showed a strong inhibition of germination to increasing salinity⁷². Coastal sand dune AM fungi are not continuously exposed to high salinities⁷². Germination of spores of *G. gigantea* was tested on immersion in seawater for 1-20 days⁷². Germination showed a significant linear decrease with the time of exposure, declined from 90% to 61% after 20 days. The germination was more reduced in the presence of chloride ions than sodium ions⁷³. The recovery of germination of AM spores was more rapid if the spores were periodically exposed to sodium ions rather than chloride ions²⁷.

Fresh roots of *Sporobolus* immersed in seawater for seven days served as AM fungal inocula in pot culture. Thus, AM fungi are capable of oceanic dispersal through the leaf sheath of litter and rhizome segments²⁷. Although a small amount of phosphorus in soil or agar improves the spore germination of AM spores, addition of KH_2PO_4 inhibits the germination of *Glomus mosseae*⁷⁴. It is suggested that a

communication mediated by volatile messengers between plant and AM fungi results in directional growth of AM spore germ tubes towards roots and roots towards AM spores^{75,76}. The colour of spores of *G. gigantea* changed from greenish-yellow to yellow to reddish-orange-brown to black (dead) due to attack of microbial parasites^{15,77}. The first two kinds readily germinated, while latter two stages did not germinate⁷¹.

Spore Ageing, Parasitism and Death

Ageing and death of AM spores on sand dunes are the common feature^{15,47,77}. Newly formed healthy spores in temperate dunes undergo ageing and death within a period of about seven months due to parasitism by microorganisms¹⁵. Climatic and other conditions affect both the onset of sporulation and rate of spore ageing. Variation in the longevity of healthy spores may be dependent on edaphic factors. Based on the studies in Rhode Island dunes, a series of stages of spore vigour of *G. gigantea* was recognized by Lee and Koske¹⁵: healthy greenish-yellow, yellow with brown spots (mottled), reddish-orange-brown and collapsed/blackened/dead. Many species of Chytridiomycetes and Deuteromycetes have been identified as hyperparasites of spores of AM fungi⁷¹. Parasitism by fungi and actinomycetes resulted in decline of AM spore abundance and reduced the inoculum potential particularly during spring and summer in Rhode Island. Parasitized spores were found throughout the year in Rhode Island dunes¹⁵. Volatile products of soil actinomycetes reduced the germination of *Gigaspora margarita*⁷¹. Forty four species of fungi and six actinomycetes have been recovered from the surface-sterilized, crushed spores of *G. gigantea*¹⁵. Greater number of pathogens/saprophytes was isolated from older spores (brown and dead) than from newly formed spores. Predation and degradation of AM fungal spores in some tropical sand dunes have been reported to be greater than in temperate dunes²⁸. The spore abundance in Hawaiian sand dunes was reduced due to microbial parasitism and predation which may favour the selection of non-sporulating AM species^{28,29}.

Dead spores of about 10 species of AM fungi of the genus *Gigaspora* have been found to be occupied by spores of other AM fungi (*Acaulospora*, *Gigaspora* and *Glomus*) in the sand dunes of Atlantic coast and Great Lakes of United States⁷⁸. Spores of 1-5 different species occurred within a dead spore. The samples of Virginia beach contained the greatest percentage of occupied spores (individual spores contained 1-100 spores). In Hawaiian dunes, *Glomus microaggregatum* occurred inside the spores of *Scutellospora hawaiiensis*²⁹. These spores would be overlooked in most collections unless larger, dead spores of *Scutellospora* are crushed and

examined²⁹. *Scutellospora hawaiiensis* was also found inside the dead spores of *Gigaspora* sp.⁷⁹. Association of one kind of AM spores with other kind of AM spores are of immense significance in terms of survival, dispersal and inoculum potential in coastal and dunes.

Effect of Edaphic Factors

Coastal sand dunes are deficient in nitrogen, phosphorus, potassium, organic matter and water³. Establishment of AM fungi in coastal sand dunes may be dependent on organic matter. Preferential association of AM fungi was found with particles of decaying organic matter^{12,18,38,80}. Organic particles in the dunes serve as energy source⁸⁰. Increased hyphal growth of AM fungi in soil with increased organic matter was evident⁸¹. The alkaline phosphatase activity by AM fungal mycelia was decreased in soil devoid of organic matter. Organic matter was expected to provide phosphorus as substrate for acid and alkaline phosphatase. Many studies in temperate locations have revealed that in sand dune ecosystem calcium and magnesium are found in adequate amounts for plant growth, while nitrogen, phosphorus and potassium are limiting⁸². Although the activities of AM fungal species are known with respect to the soil factors (heavy metals, texture, moisture, temperature, nutrient levels and salinity), the knowledge on the significance of these factors on the AM fungal diversity in natural ecosystems is inadequate⁸³. There was little correlation between the density/frequency of occurrence of AM fungal population in the soil and the physicochemical characteristics (chloride, nitrate, phosphorus, potassium, organic matter, moisture and pH) with the exception of sand grain size in Rhode Island dunes¹¹. There was no significant correlation between AM spore abundance and soil nutrients or edaphic features^{13,14}. A significant difference was found in moisture, pH, phosphate, sodium, potassium and nitrogen between naturally vegetated and non vegetated dunes of the west coast of India which may be due to the effects of dune vegetation and AM fungi³¹⁻³⁴. Among the three types of soil employed to grow the pigeon pea, the AM fungal colonization and their spore load were highest in coastal sand dune soil⁸⁴. It was related to low levels of available phosphorus in sandy soil.

Impact of Dune Disturbance

Coastal sand dunes are susceptible for a variety of disturbances like tidal action, wind blow, erosion and sand accretion. Sea erosion is a major concern in temperate and tropical regions of the world. Extensive projects have been implemented to avoid beach erosion in Atlantic coast of United States^{54,60,62,85}. In Atlantic coast besides replenishment of sand from offshore, beach grasses have been planted along the

beaches to initiate dune-building processes and thus to reduce the erosion losses. In south west coast of India, the beach areas prone to severe sea erosion are in the vicinity of coast-parallel river bends and associated coastal faults⁸⁶. The artificial granite seawall shifts the erosion laterally and thus they become ineffective⁸⁷.

Seedlings exhibits stimulation of growth, tillering, reproductive output and biomass to partial burial³. This may possibly be due to intermediate disturbance of sand dunes. The intermediate disturbance facilitates the insulation of seedling root system from high sand temperature and desiccation, escape predation from surface feeders, greater access to soil moisture, more space for root, higher nutrient in the new substrate and change in the biota resulting in an increased plant species richness and diversity^{3,34,88}. Burial of seeds, seedlings and plants in coastal sand dunes act as a strong selective force eliminates or reduces the less tolerant species and increases the abundance of tolerant species⁸⁹. Similar criteria are applicable to the AM fungal spores to establish on the sand dunes. Observation on the burial of six tropical sand dune plant species showed an increased plant vigour (biomass and leaf area) and allocation of more biomass towards the aerial parts⁹⁰. Possibly the AM fungal colonization as well as burial enhances the aerial biomass. Burial also influences the succession pattern of coastal vegetation and subsequently stabilizes the dune. Burial of *Ammophila* significantly stimulated the foliar growth rate and total plant dry mass; burial with AM fungal colonization irrespective of the presence or absence of plant parasitic nematodes elevates the foliar growth rate and total plant dry mass⁹¹. Burial of *Ammophila* also elevated the root dry mass, further elevation of root dry weight was found when plants with AM fungi was buried. When plants with AM fungi and nematodes were buried, the root dry weight was not drastically reduced, which indicate the inhibitory effects of AM fungi on nematodes.

The coastal reclamation in Singapore resulted in the establishment of non-mycorrhizal plant species due to lack of AM fungal inoculum³⁵. Severe disturbance of sand dunes reduces or destroys the AM fungal propagules which results in the reduction or elimination of plant species⁹². Such severely disturbed areas require long period for re-establishment of mycorrhizal vegetation. These situations have major impact on natural plant succession on the dunes and their stabilization.

Impact of non-AM Endophytic Fungi

The fungal endophytic association provides a competitive advantage to the coastal sand dune vegetation against herbivores and other pathogens. A few studies have been conducted to understand the interaction between mycorrhiza and other plant endophytes⁹³. Non-mycorrhizal endophytes are capable of altering the plant-plant

competition and drought resistance similar to that of mycorrhizae^{44,45}. The secondary metabolites of non-AM fungal endophytes reduce the insect herbivory⁹⁵. It is known that mycorrhizae reduce the *Acremonium*-induced insect herbivory⁹⁶. Endophytic *Acremonium* sp. has reduced the colonization and reproduction by *Glomus macrocarpum* and *Glomus mosseae* in pearl millet^{97,98}. The presence or absence of *Acremonium* can thus be employed to control the mycorrhizal community for the optimum effect on crop productivity⁹⁷. In view of the effects of non-AM fungal endophytes on AM fungi and benefits to the dune vegetation, non-AM fungi of coastal sand dunes deserve special attention.

Succession of Higher Plants in Coastal Sand Dunes

The stabilization of disturbed ecosystems particularly coastal sand dunes is dependent on the successful establishment of the most effective plant community, which is possible by the association of plant species and AM fungi⁴⁶. The dominance of non-mycorrhizal plants during the early succession has been attributed to the reduction or lack of mycorrhizal propagules after disturbance. Similar pattern of plant succession is applicable for the devastated or disturbed locations due to volcanic eruption⁹⁹ and coastal land reclamation³⁵. A survey of vegetation on the reclaimed coastal land ecosystem in Singapore after five years indicated the absence of AM fungi and the dominance of non-mycorrhizal plant species. The plant cover was reduced from >95% to <10%, while the mycorrhizal plant species was reduced from >80% to 0% towards the sea. The zone close to sea was exclusively colonized by plant species belonging to non-mycorrhizal family, Cyperaceae. Obligate mycotrophic plant species are prevented from colonizing a site if the AM fungi are absent or their abundance is below the critical level⁸⁵. But the facultatively mycotrophic non-mycotrophic plant species will be excluded when AM fungal inoculum potential is greater than the minimum level^{100,101}. Francis and Read¹⁰¹ studied the relative growth rate, survival of plant species belonging to the so-called non-host families (Brassicaceae, Chenopodiaceae and Polygonaceae) for 63 days in the presence and absence of AM fungal mycelia. These plant species showed a strong inhibition in their growth in the presence of AM mycelia, and some even suffered severe mortality. Absence of appressorium and arbuscules during the interaction between AM fungi and plant tissues suggest the lack of recognition event leading to the establishment of a functional symbiosis, instead it becomes a rather parasitic type of colonization¹⁰². Beyond the impact of AM fungi on plant growth and community structure, the role of AM fungi in coastal sand dune ecosystem dynamics has not been well understood¹⁰³.

Although the members of the family Caryophyllaceae colonize the disturbed habitats, *Silene colorata* and *S. orites* were found to be colonized in stable sand dunes without much human interference on the west coast of Italy than on the eroded and disturbed dunes by human activities¹⁶. The frequency of occurrence (60-100%) and the mycorrhizal index (1.5-3) and root colonization of AM fungi increased with the age of the substrate (8-137 years)²⁶. Similar observations were made at the volcanic sites of Mount St. Helen's mountains, United States¹⁰⁴ and Hawaiian Islands²⁶. Non-mycorrhizal plant species do not support AM fungal hyphae but may encourage the proliferation of other non-symbiotic fungal mycelia through the release of carbon compounds in the vicinity of the roots¹⁰⁵. Hence, the association and role of non-mycorrhizal fungi on sand dune plants needs more attention. The soil phosphorus was generally low in coastal sand dunes, but was significantly high at the high tide line where non-mycotrophic annuals establish including the reclaimed area¹⁰⁶. The plant succession initiation by non-mycotrophic or facultative mycotrophic species depends on the degree of disturbance and the soil fertility. Biomes with high soil fertility are usually colonized by high proportion of non-mycotrophic plant species, as in arid and semiarid lands. Succession in biomes with low fertility is initiated primarily by facultative mycorrhizal plant species, as in tropical forests or in sand dunes^{106,107}. In coastal sand dunes the extent of disturbance determines the pattern of vegetation. If the disturbance eliminates AM fungi, non-mycorrhizal plants get established, in highly disturbed conditions (erosion/accretion) all plant species may be considerably eliminated⁹¹. Thus, based on the extent of disturbance, strategies have to be developed to inoculate the AM fungi and suitable vegetation on coastal sand dunes for restoration and stabilization.

Future Outlook

Below ground diversity of AM fungi is one of the major factors governing the maintenance of plant diversity and the ecosystem functioning¹⁰⁸. The loss of AM fungal diversity decreases both plant equilibrium and ecosystem productivity, which leads to the instability of ecosystem¹⁰⁰. Although a few studies have been conducted on the AM fungi of tropical coastal sand dunes, the species richness is higher than temperate and subtropical locations. A wide variety of plant species (sedges, shrubs, herbs, climbers, creepers and tree species) which have been adapted to tropical coastal sand dunes^{27,30,31,64,66,109} offers excellent scope for re-vegetation, restoration and stabilization of coastal dunes. The mat-forming creepers (*Ipomoea pes-caprae* and *Launaea sarmentosa*) including nitrogen fixers (*Alysicarpus rugosus*, *Canavalia cathartica*, *C. maritima*), rhizomatous members of Cyperaceae and *Spinifex littoreus*

would be the ideal plant species in the restoration and stabilization of tropical coastal sand dune ecosystem. Multistoried cropping pattern on dunes would facilitate to withhold the sand movement and withstand the sand accretion. The AM fungal species which are abundant and have a wide host range (*Gigaspora margarita*, *Glomus albidum*, *G. lacteum*, *Scutellospora erythropia* and *S. gregaria*); high frequency of occurrence and wide geographical distribution (*Gigaspora gigantea*, *Glomus mosseae* and *G. dimorphicum*) would facilitate the maintenance of plant-AM fungal mutualism, enrich and restore the coastal sand dune ecosystem. Coastal sand dunes being extreme habitats provide excellent opportunities to understand the mutual interaction and adaptation of biota to environmental stress. The coastal sand dune stress-tolerant plant species and microbes deserve special attention for their application in agriculture, horticulture, forestry, environmental rehabilitation and bioremediation.

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Development of near isogenic lines of productive silkworm breeds by isozyme marker based selection

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Abstract

Of late, molecular marker facilitated investigations using isozyme/DNA markers is being widely explored in the breeding of crop plants and live stock for the improvement of desirable traits. In India, limited success of conventional breeding and selection strategies adopted so far in silkworm breeding, has warranted testing of the new strategies for maximising yield realisation. In this direction, work carried out earlier on a number of biochemical parameters has shown the prospects of using digestive amylase as a marker in silkworm breeding due to its close association with survival, better digestibility and isozyme polymorphism. A test breeding plan was adopted using the indigenous low yielding polyvoltine breeds, namely, Pure Mysore and Nistari having '4 band' and '5 band' cathodic amylase isozyme types, respectively, as donor parents (DP) and the productive bivoltine breeds like NB18, CSR2 and CSR5 with 'null' type of isozymes as recurrent parent (RP). Near Isogenic Lines (NILs) of the RPs were developed by introgressing the isozymes from the DPs via backcross breeding strategy. The evolved lines have shown significant improvement in viability over their respective RPs without any deterioration in yield traits. The laboratory evaluation has indicated superiority of a few hybrid combinations of the evolved lines over the conventional hybrids in terms of higher index values for survival and other economic traits. The methodology of the breeding scheme, the performance of the evolved lines and the future plan are discussed.

(Key words : amylase isozyme/backcross breeding/Near Isogenic Lines/silkworm)

Introduction

Sustained breeding efforts by conventional methods since the last three decades has resulted in the evolution of a number of productive Indian silkworm breeds^{1,2}. These have played a pivotal role in augmenting silk production resulting in over ten fold increase from the '60s to the '90s. In spite of achieving a quantum jump in production, the twin problems of low productivity and poor fibre quality continue to impair the vertical growth of silk industry.

To overcome these drawbacks, new breeding strategies adopted during the early '90s have resulted in the evolution of improved polyvoltine breeds like RD1, P2D1, BL lines³ as well as productive bivoltine breeds of CSR series with high shell ratio and raw silk recovery⁴ which have the potential of producing 4A international grade silk. However, the full potential of these evolved breeds has not been realised as evident from the large yield gap between the laboratory results and farmers' field which is indicated by the on-farm trials of these breeds conducted in the recent past. The possible reasons for the limited success of these breeds in the field could be the cumulative result of the interplay of poor survival potential of the new breeds, the low input-cum-low management situation prevalent at most of the farmers' places and the vagaries of the tropical environment.

Rapid advances in genetics and molecular biology have opened up new avenues for overcoming the limitations of traditional breeding methods for improving the efficiency of selection of the target trait, maximise the rate of improvement and shorten the breeding time. With regard to silkworm breeding in India, the present situation warrants testing of the novel breeding strategies which have been offered by the recent developments in molecular biology and biotechnology, for improving yield and productivity. Of late, one of these new techniques successfully being used in plant and animal breeding is marker assisted selection (MAS) employing isozyme or DNA markers for the transfer of desirable traits from phenotypically inferior parents into productive breeds.

Prospects of molecular marker assisted backcross breeding

In recent years, molecular marker facilitated backcross breeding using isozyme/DNA markers are being widely explored for introducing desirable traits in crop plants⁵⁻⁸ and live stock⁹⁻¹³.

Recurrent backcross breeding has been the method of choice for gene introduction when the recipient line (recurrent parent) has many desirable properties but lacks a specific trait that is known to be present in a donor parent. The introgression of the desired gene from the donor consists of raising the F1 between the donor and the recurrent parents. The F1 is then backcrossed to the recurrent parent and the progeny is screened with respect to the character expressed by the target gene being introduced. The process is repeated by raising several backcrosses and the population progressively becomes more like a recurrent parent.

Theoretical basis of backcross breeding

As discussed in detail by Allard¹⁴ and Welsh¹⁵, the genetic background can be considered in terms of the total information contributed by either parent. In the F₁, the genetic information from each Parent is 50%. As we begin to backcross, contribution from the donor parent (DP) is gradually increased by 50% with each backcross and is calculated by the formula $(1/2)^n$ where 'n' is the number of backcrosses. At BC₁, 75% of the genetic background would be of the recurrent parent (RP), while that of the DP would be 25%. Further, at BC₂, it would be 87.5% and 12.5% respectively. Finally at BC₁₀, the RP recovery would be about 99.95%, while the contribution of the DP is reduced to 0.05% (Fig. 1)

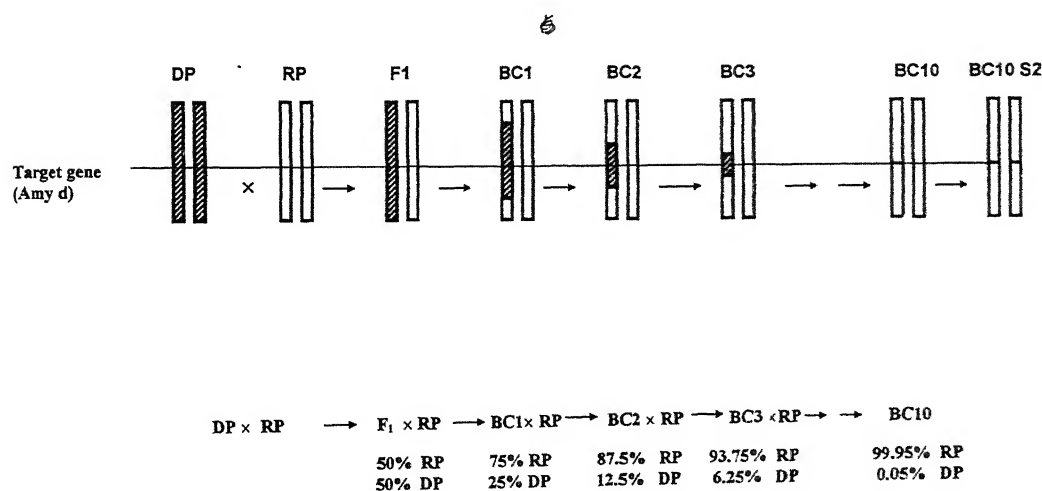


Fig. 1—Theoretical basis of backcross breeding showing the introgression of the target gene (Amy d) from the donor parent (DP) into the genetic background of the recurrent parent (RP).

Please see text for details.

Further, in the F₁, all the loci differing between the parents will be heterozygous. With backcrossing, increased homozygosity for the recurrent parent will occur. The rate at which this takes place is according to the formula : $(2m-1)/2m$, where 'm' is the

number of backcrosses. For example, after the 10th backcross, 99.9% of the segregating loci will be homozygous. Besides, if only one loci is selected from the donor parent, the proportion of genotypes homozygous would also be 99.9% as obtained by the formula $\{(2m-1)/2m\}^n$, where 'n' equals the number of heterozygous loci in the original F1 and 'm' equals the number of backcrosses.

If the parents used are quite similar genetically, minimal number of backcrosses may be sufficient for the RP recovery. In the case of genetically divergent parents, several backcrosses may be required to eliminate the undesirable genetic background of the DP. Further, linkage is another factor which affects introgression. If the target gene is linked with other undesirable genes, reduced speed of RP genotype recovery will occur. During the process of backcrossing, if the selected trait is controlled by a dominant gene, there is very little problem as selection of the progeny can be performed directly. In case of traits governed by recessive genes, alternate selfing operation has to be carried out subsequent to every backcross, to recover the homozygous recessive progeny.

After raising the required number of backcrosses, a line is obtained (that carries the target gene of the donor parent in the background) which is nearly identical to that of the recurrent parent. This line is designated as Near Isogenic Line (NIL). Most of the currently available NILs in plants have been developed by using 5-6 backcrosses. Thus a number of NILs have been generated in various crop plants as the by-products of introgression of the target gene by backcross breeding. These NILs are being successfully used to identify DNA markers that are closely linked to traits of interest¹⁶.

Identification of enzyme marker in mulberry silkworm for backcross breeding

In silkworm, earlier investigations on a number of biochemical parameters across a wide array of germplasm stocks have under scored the importance of amylase because of its significant relationship with survival and yield traits¹⁷.

Further, screening of germplasm stocks for the analysis of cathodic digestive amylase by PAGE revealed three types of isozyme polymorphisms (Fig 2). It is reported that the cathodic amylase is controlled by a single gene, 'Amy d' of the 8th linkage group (locus 2.8 cM) with three multiple alleles, i.e., Amy d^v, Amy d^{iv} and Amy dⁿ representing '5 band', '4 band', and 'null' pattern¹⁸. All the productive bivolt-

tines were of the 'null' type with very low activity, while all the polyvoltine high survival stocks were either of the '4 band' or '5 band' types having many fold high activity¹⁹.

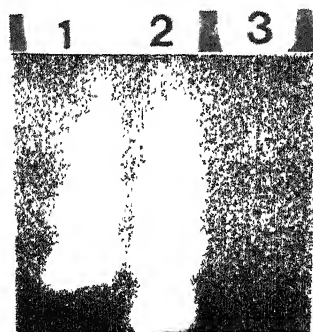


Fig 2–Digestive amylase isozyme polymorphism in silkworm stocks as revealed by cathodic PAGE. Lane 1 : '4 band' type found in Pure Mysore. Lane 2: '5 band' type of Nistari Lane 3 'null' type as shown in NB18, CSR2 and CSR5 where no bands were seen

In view of the wide genetic divergence in terms of activity and isozyme polymorphism and also because of its role in better digestibility and close association with survival, digestive amylase isozyme has been chosen as a surrogate marker in silkworm breeding for improving survival potential of the productive breeds.

Selection of parents

Indigenous polyvoltine silkworm breeds, namely, Pure Mysore with '4 band' and Nistari with '5 band' cathodic amylase types were chosen as the Donor Parents (DP). These two breeds are being reared in India for more than hundred years, the former being popular in south India and the latter in north India. The survival potential of these breeds are high which are being reared through out the year but the yield and fibre quality is poor.

Three productive bivoltine breeds, viz., NB 18, CSR2 and CSR5 with 'null type of amylase were selected as Recurrent Parents (RP). NB18 was commercialised in the early '70s but due to the low survival potential in the tropical climate, the yield realised in the field was poor and hence it is primarily being used as a male component in combination with the local polyvoltine breeds for cross breed preparation. CSR2 and CSR5 were recently authorised in 1997 for commercial use, which are highly productive with high shell ratio and superior yarn quality. These

breeds require good quality leaves and better management practices during rearing and hence presently they are being tested with selected progressive farmers during favourable seasons only. Thus, these three productive breeds were selected RPs in the present breeding scheme for improving their survival potential by using the indigenous breeds as DPs employing isozyme marker assisted selection.

The breeding scheme

A backcross breeding scheme was adopted using the Pure Mysore and Nistari which were homozygous for '4 band' (4/4) and '5 band' (5/5) amylase isozyme types respectively as donor parents (DP) and three productive bivoltine breeds, namely, NB18, CSR2 and CSR5, all with 'null' (N/N) type as recurrent parents (RP).

F1 progeny were raised between the DPs and the RPs. The F1 females which were heterozygous for the isozyme pattern (4/N or 5/N) were backcrossed to the RP (Fig 3). The BC1 progeny were again backcrossed to the RPs. In BC2, two types of beds were expected. In one type, 50% of the progeny heterozygous (4/N or 5/N) and the other 50% homozygous for the 'null' (N/N) pattern. In the other type of bed, all the progeny (100%) to be homozygous for the null (N/N) pattern. In order to select the individuals having the donor isozyme pattern, the BC2 progeny were reared in 30 cellular beds and the progeny were electrophoretically screened bedwise and the progeny showing the heterozygous (4/N or 5/N) pattern were selected and backcrossed again to the RP. The progeny showing homozygous pattern for the null (N/N) were culled.

The procedure was repeated and ten backcrosses were made with the RP. At BC10, the progeny were still in the heterozygous condition for the amylase isozymes, i.e., 4/N or 5/N and hence they were selfed to obtain the homozygous pattern (4/4 or 5/5) of the donor isozymes. Hence, the BC10 progeny were subjected for individual screening for the isozyme and the progeny (4/N or 5/N) were selected and BC10 S1 generation was raised. In the BC10 S1 bed, 25% of the progeny were homozygous (4/4 or 5/5) and 50% will be heterozygous and the remaining 25% homozygous for the 'null' (N/N) type.

During electrophoresis, the homozygous (4/4 or 5/5) could not be distinguished from the heterozygous (4/N or 5/N) pattern in the gel. Hence, in order to select the homozygous progeny, a second selfing was required and followed by a test cross with N/N for distinguishing homozygotes from heterozygotes.

So, the BC10 S1 progeny were subjected for enzyme analysis individually and the progeny showing the 4 band or 5 band phenotypes were selfed and BC10 S2 families were raised and reared as 30 cellular beds. In each bed 10 batches of BC10 S3 progeny were raised by random mating and the layings were hibernated. Further, test cross progeny of BC10 S2 x RP were also raised in each bed which were reared cellularly. Each bed of the test cross progeny were again individually screened enzymatically and the progeny having all individuals of 4 and 5 band phenotypes were identified and the corresponding BC10 S3 progeny with the homozygous (4/4 or 5/5) pattern of the donor isozymes were selected and retained.

Thus, three Near Isogenic Lines, namely, NNB (NIL of NB18) and NC2 (NIL of CSR2) with 4 band pattern and NN1 (NIL of CRS5) with the 5 band pattern were derived by introgressing the isozymes from the multivoltine donors i.e., Pre Mysore and Nistari respectively.

Performance of the evolved lines

The rearing performance of backcross generations NNB, NC2 and NNI lines from BC1 to BC9 as well as the selfed generations from BC3 to BC10 vis-a-vis their respective recurrent parents under seventeen crops between April 1995 and June 99 period is depicted in Table 1. In these generations, only few progeny were sampled showing the isozyme type of the DP and the entire beds were selected and continued and the rearing data are available. While, the selfed generations at BC10, BC10 S1, BC10 S2, which was followed by a testcross, only about 100 female and male larvae each were subjected for individual screening for isozyme type of the DP and selfed, hence, the rearing data are not available. Perusal of the data of NILs reveal that the evolved lines have consistently performed well in all the generations in comparison to their respective RPs. The evolved lines have also shown improvement in survival (represented as Yield/10000 larvae by number), at the same time maintaining the cocoon characteristics on par with those their recurrent parents.

The mean estimates of the rearing data of the evolved lines and their respective RPs were subjected for 't' test which revealed significant increase in survival in the evolved NILs over RPs (Table 2). In case of cocoon traits, though marginal decrease was noticed in the NILs, the differences were not significant except for shell ratio in NC2.

Gen.	Season	Yield/10000 larvae By No.	By wt. (Kg)	CWT (g)	SWT (g)	SR (%)	Gen.	Yield/10000 larvae By No.	By wt. (Kg)	CWT (g)	SWT (g)	SR (%)
NIL : NNB												
RP: NB18												
BC1	Apr.95	9599	15.6	1760	0.331	18.8	1	9320	14.8	1656	0.363	21.9
BC2	Jun.95	8391	12.3	1540	0.290	18.8	2	7447	8.8	1420	0.280	19.7
BC3	Aug.95	8290	13.7	1780	0.347	19.5	3	7866	11.6	1600	0.309	19.3
BC4	Oct.95	9196	14.3	1629	0.315	19.3	4	8640	13.9	1740	0.340	19.5
BC5	Dec.95	8655	11.6	1390	0.260	18.7	5	8567	14.8	1480	0.274	18.5
BC6	Feb.96	8785	11.4	1355	0.263	19.4	6	7408	11.3	1522	0.301	19.8
BC7	Apr.96	8280	14.3	1422	0.281	19.8	7	8083	9.8	1289	0.236	18.3
BC8	Jun.96	8360	11.9	1565	0.314	20.1	8	7540	10.8	1547	0.314	20.3
BC9	Aug.96	9400	14.7	1571	0.301	19.2	9	8653	13.5	1768	0.322	19.2
BC10 S3	Sep.97	9086	13.6	1518	0.291	19.2	10	8266	13.0	1612	0.316	19.6
BC10 S4	Nov.97	8593	13.9	1697	0.338	19.9	11	8067	14.6	1886	0.362	19.2
BC10 S5	Jan.98	9100	14.3	1531	0.318	20.8	12	8664	15.6	1707	0.350	20.5
BC10 S6	Mar.98	8552	12.1	1407	0.288	20.5	13	8316	13.1	1578	0.320	20.3
BC10 S7	Jun.98	7293	10.2	1362	0.258	18.9	14	7120	9.23	1293	0.267	20.6
BC10 S8	Aug.98	8814	12.9	1481	0.290	19.6	15	7568	12.1	1583	0.297	18.8
BC10 S9	Oct.98	8547	13.3	1440	0.264	18.3	16	7760	12.2	1567	0.289	18.4
BC10 S10	Dec.98	9413	17.1	1795	0.374	20.8	17	9000	16.0	1824	0.364	20.0
Mean		8727	13.4	1544	0.301	19.5		8132	12.6	1587	0.312	19.6
SD		540	1.7	0.141	0.032	0.7		602	2.1	0.157	0.035	0.9
NIL : NC2												
RP: CSR2												
BC1	Jun.95	9000	14.4	1580	0.330	21.5	1	6080	8.3	1390	0.322	23.2
BC2	Aug.95	8877	16.0	1620	0.362	21.0	2	8288	15.3	1910	0.397	20.8
BC3	Oct.95	8440	14.8	1762	0.372	21.1	3	7576	12.5	1590	0.386	22.1
BC4	Dec.95	8530	13.0	1580	0.329	20.8	4	8133	14.3	1580	0.322	20.4
BC5	Feb.96	9164	13.2	1453	0.309	21.3	5	8133	12.5	1575	0.357	22.7
BC6	Apr.96	8944	17.4	1333	0.286	21.5	6	8173	10.5	1334	0.286	21.4
BC7	Jun.96	8968	14.3	1598	0.336	21.0	7	8240	11.9	1678	0.360	21.5
BC8	Aug.96	9170	15.7	1743	0.377	21.6	8	8816	16.0	1800	0.396	21.9
BC9	Oct.96	8980	15.9	1830	0.405	22.1	9	8640	15.5	1875	0.422	22.5
BC10 S3	Nov.97	8773	16.6	1952	0.417							

Table 1-Continued.....

Gen.	Season	Yield/10000 larvae By No.	By wt. (Kg)	CWT (g)	SWT (g)	SR (%)	Gen.	Yield/10000 larvae By No.	By wt. (Kg)	CWT (g)	SWT (g)	SR (%)
NIL : NN1												
BC1	Oct.95	9700	18.1	1.885	0.382	20.3	1	8512	15.8	1.872	0.407	21.7
BC2	Dec.95	9066	14.9	1.680	0.313	18.6	2	8480	14.4	1.680	0.331	19.7
BC3	Feb.96	8825	13.3	1.507	0.315	20.9	3	7184	11.8	1.652	0.355	21.5
BC4	Apr.96	8580	11.7	1.369	0.287	21.0	4	8040	10.2	1.354	0.286	21.1
BC5	Jun.96	9088	13.3	1.521	0.307	20.2	5	8651	14.0	1.633	0.353	21.6
BC6	Aug.96	9260	14.7	1.644	0.326	19.8	6	9500	16.8	1.744	0.354	20.3
BC7	Oct.96	8600	13.9	1.647	0.341	20.7	7	7925	13.5	1.794	0.372	20.7
BC8	Nov.97	9708	14.7	1.731	0.364	21.0	8	8373	14.0	1.699	0.340	20.4
BC9	Jan.98	9381	14.5	1.518	0.324	21.3	9	9320	15.5	1.603	0.330	20.6
BC10 S3	Mar.98	8452	12.9	1.543	0.343	22.2	10	8320	12.1	1.482	0.344	23.2
BC10 S4	Jun.98	7757	10.1	1.250	0.254	20.3	11	7168	9.4	1.305	0.285	21.8
BC10 S5	Aug.98	8154	11.2	1.473	0.310	21.0	12	7488	10.1	1.454	0.315	21.7
BC10 S6	Oct.98	7777	13.1	1.582	0.321	20.3	13	7555	12.2	1.576	0.318	20.2
BC10 S7	Dec.98	8907	17.2	1.918	0.423	22.1	14	8880	16.6	1.919	0.443	23.1
BC10 S8	Feb.99	9460	15.9	1.696	0.361	21.3	15	9016	14.4	1.563	0.347	22.2
BC10 S9	Apr.99	7680	9.7	1.313	0.282	21.5	16	7520	9.8	1.407	0.308	21.9
BC10 S10	Jun.99	8052	11.5	1.514	0.319	21.1	17	7560	11.6	1.521	0.329	21.6
Mean		8732	13.6	1.576	0.320	20.8		8207	13.1	1.602	0.342	21.4
SD		655	2.2	0.174	0.039	0.039		706	2.3	0.168	0.038	0.9

Gen = Generation; CWT = Cocoon weight; SWT = Shell weight; SR% = Shell ratio % (SWT x 100/CWT)

Table 2—Results of 't' test between the evolved NILs their respective RPs.

< -----t value----- >							
NILs	vs	RPs	Yield (By No.)	Yield (By wt.)	Cocoon wt.	Shell wt.	Shell Ratio
NNB	vs	NB18	3.032**	1.112(NS)	-0.849(NS)	-0.914(NS)	-0.508(NS)
NC2	vs	CSR2	3.221**	1.108(NS)	-0.458(NS)	-1.496(ns)	-3.849***
NN1	vs	CSR5	2.250*	0.637(NS)	-0.439(NS)	-1.096(NS)	-1.922(NS)

*, ** and *** denote significance of 't' at 5%, 1% and 0.1% respectively, NS = Not significant.

For evaluation of these evolved NILs and also to identify promising hybrid combinations for further trials, eight hybrid combinations were prepared along with control hybrid KA x NB4D2 which is the traditional bivoltine commercial hybrid reared by the farmers since the last two decades. These hybrids were reared under six trials during 1998-99 period. From the rearing performance of these hybrids under six trials (Table 3), it is evident that all the eight hybrids have shown improvement over the control not only in survival (Yield/10000 larvae), but also on productivity traits like shell wt., shell ratio, filament length, renditta, raw silk % etc. Further, for identifying the potential hybrid combinations, they were evaluated by multiple trait index. For calculating the index values, the negative traits like total larval duration, V age larval duration, denier and renditta, where lesser values are desirable have been omitted and the remaining nine traits were included. Evaluation index (E.I.) was calculated²⁰ for each trait of each hybrid as per the following formula :

$$E.I. = \frac{A-B}{C} \times 10 + 50$$

- Where, A = value obtained for a particular hybrid for particular trait
 B = Mean value for a particular trait of all hybrids
 C = Standard deviation of a particular trait for all hybrids.
 10 = Standard unit
 50 = Fixed value

Table 3.— Rearing performance of hybrids developed by isozyme selection (Mean of six trials — May '98 to April '99)

SL. NO.	HYBRID	Eggs/DFL	Total L. D. (Hours)	V Age L. D. (Hours)	Yield/10000 larvae By No. By wt. (Kg)	Cocoon weight (g)	Shell weight (g)	Shell Ratio (%)	F. L. (m)	Denier	Ren-ditta	Raw Silk (%)	Ree-labi-lity
1	NC2 x NN1	484	492	136	9320	1.793	0.384	21.4	1037	2.40	6.50	16.0	82.0
2	NC2 x CSR5	462	496	140	9044	1.800	0.391	21.7	979	2.55	6.33	16.1	88.3
3	NC2 x NNB	462	490	139	9107	1.736	0.363	20.9	902	2.40	6.51	15.8	84.2
4	NC2 x NB18	473	485	136	9196	1.774	0.373	21.0	932	2.40	6.50	15.6	85.4
5	CSR2 x NN1	492	489	137	9458	1.806	0.404	22.4	1014	2.47	6.39	16.3	83.4
6	CSR2 x CSR5	499	496	138	9129	1.789	0.406	22.7	1099	2.49	6.05	16.9	80.2
7	CSR2 x NB18	504	489	133	9189	1.796	0.383	21.3	1011	2.58	6.44	15.9	83.0
8	CSR2 x NNB	522	488	135	9403	1.796	0.385	21.5	947	2.41	6.38	16.1	80.1
9	KA x NB4D2	522	494	133	9062	1.841	0.360	19.5	966	2.23	7.23	14.4	81.4
	(Control)												
Mean		491	484	136	9212	1.792	0.383	21.4	987	2.44	6.48	15.9	88.9
SD		22	20	2	141	0.026	0.015	0.9	57	0.10	0.29	0.6	1.6

DFL = Disease Free Laying L.D. = Larval Duration F.L. = Filament Length

Mean index values were calculated for the nine traits for the nine hybrid combinations reared under six trials. The index values for survival as well as the overall index values for nine traits (Table 4) indicate that for survival, except NC2 × CSR5, the remaining seven hybrid combinations have scored higher index values over the control hybrid, KA × NB4D2 and five hybrids have higher values than the hybrid of the Recurrent Parents, i.e., CSR2 × CSR5. Further, in terms of overall index values for nine traits, seven hybrids were found to be superior over the control. The hybrid NC2 × NN1, where both the parents have been introgressed with isozymes from the DPs, the hybrids, CSR2 × NN1 and CSR2 × NNB, where one of the parents were of the isozyme type of the DPs have scored higher overall index values. Among all the hybrids, CSR2 × NN1 has scored the highest index values for survival (67.5) as well as all the nine traits (58.6) exceeding the values of not only the control hybrid but also CSR2 × CSR5. It is interesting to note that in the hybrid CSR2 × NN1, CSR2 is of 'null' and NN1 is of the '5 band' type of amylase. This indicates the possibility of higher heterotic vigour of 'null' and high enzyme activity combinations than the others. Even though at the laboratory level, other than CSR2 × NN1, the remaining hybrids having parents introgressed with isozymes from the DPs have not outscored the values of CSR2 × CSR5, the differences could be more pronounced under the field situation.

Table 4—Mean Index values of the hybrids (average of six trials)

Sl. No.	Hybrids	Index Value for	
		Survival	All traits
1	NC2 x NN1	57.7	52.3
2	NC2 x CSR5	38.1	49.7
3	NC2 x NNB	42.5	40.8
4	NC2 x NB18	48.9	45.6
5	CSR2 x NN1	67.5	58.6
6	CSR2 x NB18	48.4	51.8
7	CSR2 x CSR5	44.1	54.9
8	CSR2 x NNB	63.6	52.7
9	KA x NB4D2 (Control)	39.3	43.6

It is expected that few of the new hybrids developed by isozyme selection may perform better than the existing hybrids in the field. Hence it has been planned to conduct multilocal trials of few of these hybrids selected on the basis of index values, at regional research stations during 1999-2000 for identifying the promising hybrid combinations for large scale field testing.

Concluding Remarks

Molecular marker assisted backcross breeding is being successfully used for introducing desirable traits from the donor to the recipient line and as the offshoot of this breeding process, a number of NILs have been developed in various crop plants and live stock. It has also been reported that phenotypically inferior wild varieties when crossed with the high yielding domesticated varieties have shown transgressive variation²¹ which has been used to improve many traits of economic importance. Wild relatives have been exploited as a source of disease and insect resistance in many crops but their use for the transfer of this trait is less attractive during breeding as the transfer of the target trait is often accompanied by many linked undesirable characters. This phenomenon is referred to as 'Linkage drag'²². Thus intensive selection has to be carried out to recover the productive lines. However, it has been shown that using molecular marker assisted selection, this linkage drag can be minimised^{5,8}.

In the case of silkworm also, earlier breeding programmes using the indigenous poor yielders like Pure Mysore and Nistari as one of the parents has resulted in limited improvement in the evolved lines when phenotypic selection alone was employed. In the present breeding scheme, even though low yielding parents have been used as donors, selection based on amylase isozymes has not resulted in any deterioration in the cocoon traits in the evolved lines, while there has been significant improvement in the survival potential.

Since survival is a complex trait, it is not possible to substantiate how selection of '4 band' or '5 band' amylase isozymes with high activity is directly responsible for high survival. However, earlier it has been shown that races with high amylase content could digest and survive better when poor quality leaves or artificial diet was fed when compared to races with low amylase content²³. Further, low amylase activity stocks were found to excrete five fold more starch than the polyvoltine stocks with high amylase activity²⁴. It means that the former cannot digest and assimilate starch adequately which may be affecting the carbohydrate metabolism, which in turn could result in lower fitness by some pathway which is not known presently and further in depth studies are needed to support this contention. It is believed that increased enzyme activity and efficient starch digestion may confer better adaptability under the

tropical condition here the leaf quality is generally poor and hence could result in better survival.

Gimelfard and Lande²⁵ have suggested the potential utility of combining molecular information with phenotypic selection for improving the efficiency of selection and maximising the rate of improvement of the selected trait. Thus the new strategy employed in the present study by integrating isozyme based selection with the conventional phenotypic selection has clearly shown the prospect of its use in silkworm breeding.

In the recent years there has been rapid developments in genome analysis of silkworm and the RAPD/RFLP maps have already been published^{26,27}. Application of PCR based RAPD and also DNA finger printing with minisatellite probes have revealed distinct DNA profiles between diapausing and non-diapausing stocks^{28,29}. It is likely that in the near future a number of molecular markers will be identified which are linked to traits of commercial importance in silkworm. In the foreseeable future, the molecular marker assisted selection might play a key role in the improvement of silkworm breeds.

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Induction of premature mitosis in BHK cells blocked in S-phase

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Abstract

Induction of premature mitosis and its progression were studied in 2-aminopurine treated BHK₂₁ cells blocked in S-phase by prior treatment with hydroxyurea. The cells showed premature induction of mitosis with nuclear envelope breakdown and chromosome condensation in general, and failed to progress through metaphase and anaphase. Immunofluorescence studies using anti-beta-tubulin antibody revealed normal spindle formation, but the chromosomes were not aligned to the spindle. Immunofluorescence and immunoelectron microscope studies using antikinetochore antibody showed that kinetochores got detached from the clumped and entangled chromosome mass possibly due to pull by the spindle fibres. Moreover during 2-AP treatment in S-blocked BHK cells no new cyclin is being synthesized and the initiation of mitosis is caused possibly due to activation of accumulated inactive mitosis promoting factor (MPF).

(**Keywords** : premature mitosis/metaphase/anaphase/animal & plant cells)

Introduction

Mitosis depends on the completion of S-Phase. However, Schlegel and Pardee¹ were first to report that this dependence can be overcome in BHK-cells by some purine derivatives which likely act as tyrosine kinase inhibitors². Earlier several genetical mutants capable of overriding S-Phase dependency were reported^{3,4}. Subsequently induction of premature mitosis has been reported in a number of animal cells^{1, 5-9}. We demonstrated¹⁰ that caffeine and 2-aminopurine (2-AP) could induce premature mitosis in S-blocked root meristematic cells of *Allium cepa*, indicating some common biochemical pathways leading to mitotic initiation in plant and animal cells. Schlegel and his co-workers⁷ reported premature induction of mitosis in 2-AP treated BHK (baby hamster kidney) cells. Andreassen and Margolis¹¹ noted partial mitosis in these cells without functional spindle and thus the cells failed to enter metaphase or anaphase. Earlier Brinkley and co-workers¹¹ observed detachment of kinetochores from chromosomes in such prematurely induced mitotic cells. In onion

cells we¹⁰ observed regular mitosis with functional spindle but with extensive fragmentation of chromatics at anaphase stage. This led us to undertake experiments using BHK₂₁ cells to understand how the mitotic progression differed in animal and plant cells. Our results have been discussed in relation to our observation in onion cells to explain the difference in the mitotic progression in these two different cell types.

Materials and Methods

Baby hamster kidney (BHK₂₁) cell were used as material. Cells grown on tube slips were used for light microscopic and immunofluorescence studies. Cells were synchronized by treating them with 2.5 mM hydroxyurea (HU)¹³ for 3 hrs. To induce premature mitosis BHK₂₁ cells were treated with 2-aminopurine (Sigma) for different time periods at a concentration of 1 mM. For light microscopy cells were fixed in a solution of formaldehyde, acetic acid and alcohol mixed in the proportion of 15:1:85 and stained with haemalum. Cell spreads were prepared by treating them with 0.075 M KCl for 20 mins. Chromosomes were stained with Giemsa solution.

For light microscopic immunofluorescence studies cells were fixed in 4% freshly prepared paraformaldehyde and quenched in 50 mM NH₄Cl solution and permeabilized in 0.3% Triton-X-100 (containing 0.1% BSA and 0.1 gelatine dissolved in PBS) for 5 to 10 mins (for details see¹⁴). For localisation of spindle microtubules anti Beta-tubulin (1:100, monoclonal, Amersham) and Rhodamine linked goat anti-mouse IgG (1:50, Dianova) were used as primary and secondary antibodies. For detection of centromeres antibody obtained from a CREST patient was used in dilution 1:5000. Rhodamine linked anti-human antibody (Dianova) was used as secondary antibody. For detection of mitotic cyclin, anti-cyclin B₁ (1:5, monoclonal, Oncogene Science) and Rhodamine conjugated goat anti-mouse- IgG (1:50, Dianova) were used.

For immunoelectronmicroscopy cells were fixed in 4% paraformaldehyde for 10 mins, quenched in 50mM NH₄Cl. Then the immunoreactions were performed. Anticentromeric antibody obtained from the serum of a CREST patient was used as the primary antibody (for 2 hrs.). 5 nm nanogold labelled antihuman IgG, raised in goat was used as secondary antibody (Nanoprobes INC, USA, for details of methods see¹⁵).

For immunoblot preparations cells were harvested both from the S-blocked and 2-AP treated cultures. Monoclonal anticyclin B₁ (Oncogene Science) was used as primary antibody and alkaline phosphatase conjugated goat anti-mouse IgG (Dianova) was used as secondary antibody. For details of procedure see¹⁴.

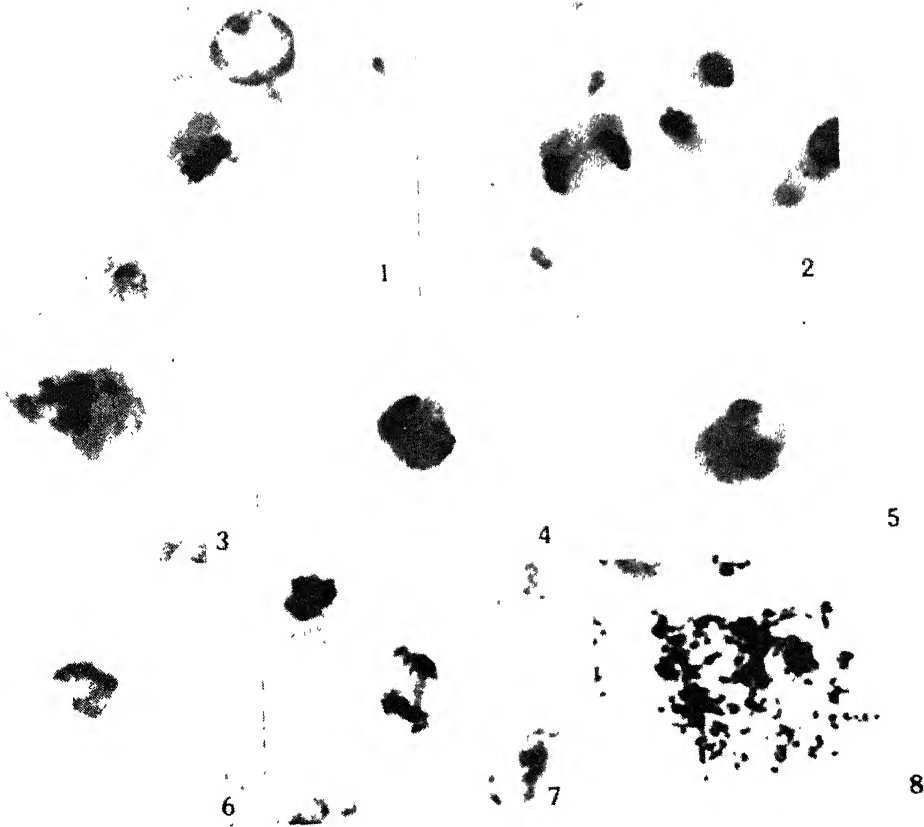


Fig. 1-2 : Control preparations from normal BHK₂₁ cells.

1—a metaphase plate with chromosomes arranged at the equatorial plate.

2—cell in early and late telophase.

Fig. 3-8 : Cells blocked in S-phase induced to premature mitosis treated with 2-AP.

3—condensed chromosomes in a single clump.

4 and 5—showing more than one clump.

6—chromosomes in a single clump-note small bits of chromatin outside.

7—an anaphase appearing at the early stages of treatment.

8—a spread preparation from a prematurely induced mitotic cell-note thicker and thinner regions of the chromosomes.

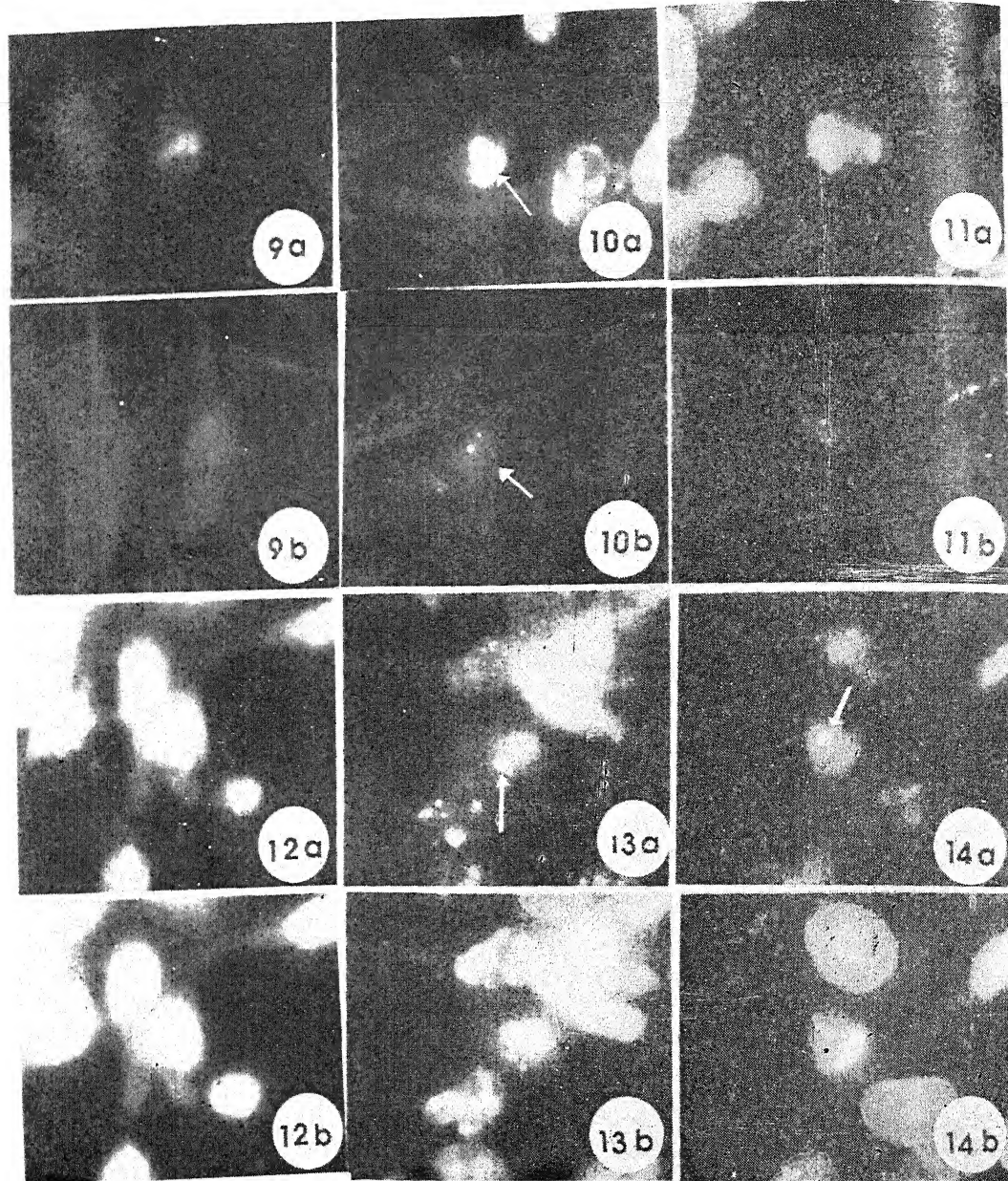


Fig. 9 : A normal mitotic cell immuno-stained for β -tubulin

(a) mitotic spindle (b) stained with Hoechst 33258.

Fig. 10 and 11 : Prematurely induced mitotic cells (a) showing mitotic spindle, (b) the same cell stained with Hoechst-note orientation of the spindle outside the main chromosome mass, only small bits of chromatin can be found within the spindle.

Fig. 12 : A normal mitotic cell

(a) immunostained for kinetochores

(b) the same cell, chromosomes stained with Hoechst.

Fig. 13 and 14 : Prematurely induced mitotic cells

(a) showing kinetochores

(b) the same cells stained with Hoechst-note kinetochores are located outside the main chromosome mass.

Results

The cells treated with hydroxyurea for 3 hrs. showed about 60% cells in S-phase (determined by flow cytometry-data not presented). These treated cells when allowed to recover in fresh medium free of drug showed normal mitosis after 5-6 hrs. of recovery. On the contrary, when the cells were continuously treated with HU for this period, no mitotic cell could be observed. However, the cells which were treated with HU for 3 hrs and subsequently treated with 2-aminopurine (2-AP) along with HU for different periods showed mitosis at different frequencies. The frequency increases with the period of treatment with 2-AP. Further experiments were done with cells treated for 8 hrs. which showed maximum frequency of mitotic cells (about 6%).

Light microscopic observation from haemalum stained control preparations revealed normal mitosis (4.2 %) with normal mitotic configurations (Figs. 1 and 2). Regular cytokinesis could also be observed. But in S-blocked cells treated with 2-AP, some of them rounded up and entered into mitosis. Condensed chromosomes could be seen in one (Fig. 3) or more clumps (Figs. 4 and 5), sometime bits of chromatin could be found in between. Small bits of chromatin could also be observed outside a prominent single chromosome clump (Fig. 6). Occasional metaphase and anaphase configuration could be found at the early period of treatment (3 to 4 hrs) (Fig. 7), but at later stages, such configuration could not be observed.

These mitotic cells in S-blocked population were induced to mitose prematurely became evident from spread preparations (Fig. 8). Thicker and thinner regions may represent the replicated and unreplicated regions of the chromosomes.

The spindle morphology was studied by indirect immunofluorescence. Figs. 9(a, b) show normal spindle formation in a control cell. In treated cells the chromosome masses were found outside the spindle, but the spindles seemed to be in normal position. Only a small mass of chromatin could sometime be found within the spindle (Figs 10, 11).

The control preparations for immunofluorescence studies on kinetochores showed kinetochores in association with the chromosome mass as expected (Fig. 12). In prematurely induced mitotic cells the kinetochores were seen not in physical contact with the main chromosome mass (Figs. 13, 14) but in association with small bits of chromatin within the spindle region. The kinetochores seemed to be detached from the chromosomes. This can be better substantiated from immunoelectron microscopic pictures. Immunolabelled kinetochores can be seen outside main chromatin mass but associated with small chromatin bits aligned to the spindle poles (Fig 15). It can be noticed that some of the kinetochores have even moved to the poles (Fig. 16).

Immunoblots from the protein samples showed that the antibody had a higher affinity for the HeLa cyclin than the BHK₂₁ cells. This is expected as the antibody was raised against human cyclin B₁. In BHK₂₁ cells, the S-blocked cells and those subsequently treated with 2-AP did not show any difference in the cyclin B₁ level (Fig. 17) indicating no new synthesis of cyclin B₁ in the 2-AP treated cells.

Discussion

2-AP induces premature mitosis in BHK₂₁ cells blocked in S-phase :

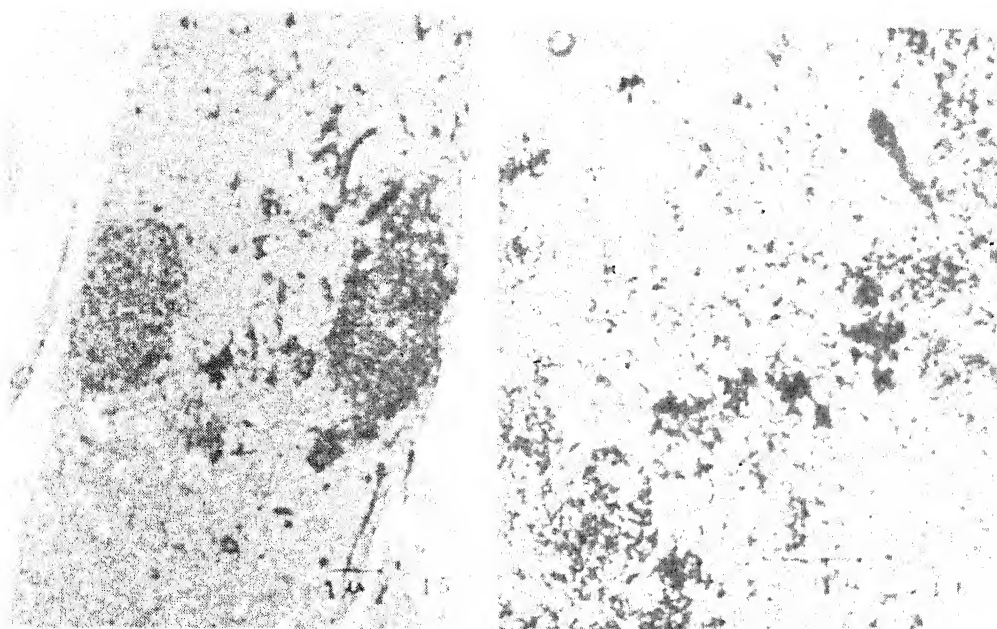
Baby Hamster kidney (BHK₂₁) cells treated with 2-AP in presence of hydroxyurea show premature mitosis in about 6% cells. The chromosomes from spread preparations show fuzzy, pulverized appearance comparable to that observed by Rao and Johnson¹⁶ in their experiments fusing mitotic cells with S-phase cells. These prematurely induced mitotic cells show cytoplasmic 'rounding up' and nuclear envelope breakdown. Mitotic progression can be noted only in few cells which enter mitosis in early phase of treatment, but the cells entering mitosis later can not progress through metaphase and anaphase. Chromosomes formed clumps possibly due to stickiness of unreplicated chromosome segments and breaks caused by spindle pull.

Abnormal alignment of Chromosomes in relation to spindle :

2-AP induced premature mitotic cells exhibit chromosome condensation and other mitotic features, but the cells fail to progress through mitosis (also reported earlier^{11,17}). However, spindle formation is normal in these cells, as can be seen by immunofluorescence study using beta-tubulin antibody. Clumped chromosome masses are found outside the spindle region. Further studies using antikinetochores antibody at the light microscope and electron microscope level reveal kinetochores with fragments of chromatin detached from the main chromosome mass. These kinetochores, however, are arranged within the spindle. Some of them even move towards the pole. Brinkley and co-workers¹² also observed this phenomenon.

No change in cyclin B level in cells blocked in S-phase and in cells subsequently treated with 2-AP :

Immunoblot results show that there is no significant variation in cyclin B level in HU blocked cells and cells treated simultaneously with 2-AP for another 8 hr. Earlier Steinmann and co-workers¹⁸ noted that cyclin B synthesis was completed within S-phase in BHK cells. This is in agreement with present observations. In onion cells along with dephosphorylation of inactive MPF further synthesis of cyclin B is



66

45

29

M

3

2

1

17

Fig. 15 : An electromicrograph of a prematurely induced mitotic cell in low magnification-note two main chromosome groups lying outside and the spindle with two centrioles lying in between. Bits of chromatin with detached kinetochores are attached to spindle fibres (arrows). Arrowhead points to a detached kinetochore almost reaching the pole.

Fig. 16 : Electron micrograph of a prematurely induced BHK₂₁ mitotic cell at higher magnification showing gold labeling of the detached kinetochores (arrow).

Fig. 17 : Immunoblot analysis of cyclin B of whole cell extract of BHK₂₁.

Lane 1 – HeLa cells

Lane 2 – S-blocked cells treated with 2-AP for 8 hrs.

Lane 3 – Cells blocked in S-phase

M–Molecular weight markers.

necessary to initiate premature mitosis. But in BHK cells there is no significant amount of new cyclin B synthesis in 2-AP treated cells. Perhaps there is only activation (dephosphorylation) of accumulated MPF which leads to the induction of premature mitosis in these cells.

Kinetochore detachment is a consequence of premature induction of mitosis in S-blocked cells :

A few BHK₂₁ cells show normal progression of mitosis in early period of 2-AP treatment, but afterwards these premature induced mitotic cells fail to show mitotic progression. These chromosomes form clumps and the kinetochores get detached possibly due to pull of the spindle fibres. Some of the detached kinetochores can even reach the poles. This is in contrast to what we observed in onion cells. Perhaps in *A. cepa* only late S-phase cells could enter premature mitosis. As such those cells could progress through mitosis, although high degree of chromosomal breaks could be observed in anaphase, due to failure of separation of sister chromatids in unreplicated regions of chromosomes¹⁰.

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***Zanthoxylum alatum* extract as mosquito larvicide**

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Abstract

Petroleum ether extract of *Zanthoxylum alatum* showed significant insecticidal activity against late third instar larvae of mosquito, *Culex pipiens quinquefasciatus* Say, LD₅₀ of its active principle (fatty acids) for third instar mosquito larvae was found to be 20.45 ppm.

(Key word : toxicity/*Zanthoxylum alatum*/extraction/bioassay/fatty acids.)

Introduction

Chemical insecticides have been used indiscriminately during the past few decades to control the pests. This has produced serious repercussions such as pest resistance, mammalian toxicity, bio accumulation and environmental damage¹. There are a number of plants, which have unique capacity to biosynthesize chemicals that are toxic to pests. Such Eco friendly phyto chemicals are termed as "bio pesticides" and the same are employed in the integrated pest management ^{2a, 2b}. We have been screening a number of plants as part of our endeavor to evaluate and identify bio active compounds. During bio-assay directed investigation on *Zanthoxylum alatum* (N.O. Rutaceae), we have identified an active fatty acid component from the petroleum ether extract which showed significant toxicity against larvae of mosquito, *Culex pipiens quinquefasciatus* Say. The present communication describes for the first time the isolation, identification, characterisation and evaluation of the insecticidal component of *Zanthoxylum alatum*.

Materials and Methods

Bioassay : A standard culture of mosquito was maintained in the laboratory at 27 ± 1°C and 75 ± 5% RH. Extract was solubilised in a minimum amount of acetone. Water samples (50 ml) were treated with varying concentrations of *Zanthoxylum*

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alatum extract (50 to 400 ppm) and its fractions (varying from 10 to 75 ppm) were used for bioassay. About 20 third instar larvae were added in 50 ml of treated water for 24 hr. and insecticidal action was assayed by scoring percent mortality of the larvae. The experiments were repeated for 4 times with 20 larvae per replicate. Percent larval mortality was corrected using Abbott's formula³. The methods adopted for testing the extracts are based on procedures recommended by the World Health Organization which are basically modified Busvine and Nash method (1953) to score insecticidal activity⁴.

Extraction and isolation of the insecticidal principle : The ground seeds (200 g.) of *Zanthoxylum alatum* were extracted with petroleum ether (60-80°) in a Soxhlet apparatus. The solvent was removed under vacuum to yield an oily residue (34 g.). The defatted seeds were further extracted with chloroform followed by the extraction with methanol. The above extracts were evaporated in vacuum to obtain respective crude extracts. A part of the residue of the petroleum ether extract (5 g.) was dissolved in ether and extracted with aqueous sodium hydroxide solution (2%). The aqueous phase was acidified with dilute hydrochloric acid and extracted with chloroform. The latter extract was washed with water, dried over anhydrous sodium sulphate and evaporated in vacuum to yield a mixture of fatty acids (1.8 g.). A part of this acid mixture was esterified with dry methanol and *p*-toluene sulfonic acid. The methyl esters were analyzed by GLC. The major components were identified as the esters of palmitic (16:0), 10.5%; palmitolic (16:1), 31.47%; *cis*-10-octadecenoic (18:1), 25.5%; *cis*-9,12-octadecadienoic, (18:2), 18.76% and *cis*-9,12,15-octadecatrienoic, (18:3), 12.65%, acids.

The toxicity studies were carried out with this fatty acid mixture obtained from the petroleum ether extract.

Results and Discussion

Table 1— Toxicity of extracts/fractions from *Zanthoxylum alatum* against third instar larvae of mosquito, *Culex pipiens quinquefasciatus* Say

Extracts/fractions	LD ₅₀ value (ppm)
Petroleum extract	315.50
NaOH extract	145.65
Fatty acid mixture	020.45

Zanthoxylum alatum is reported to have anti fungal⁵, anti microbial⁶, anthelmintic⁷ and other medicinal properties⁸. In this paper for the first time its insecticidal activity against the mosquito larvae has been revealed. Fractionation protocol for the isolation of the insecticidal principle of this plant is presented in Fig.1. The LD₅₀ value of each fraction has been presented in Table 1. It was observed that the portion containing fatty acid exhibited higher insecticidal activity against mosquito larvae as compared to other fractions. Upon probit analysis of this data, the LD₅₀ was determined to be 20.45 ppm for this fraction. There are a number of reports such as oleic, caproic and octanoic acids which exhibit significant ovicidal as well as insecticidal activities⁹⁻¹¹.

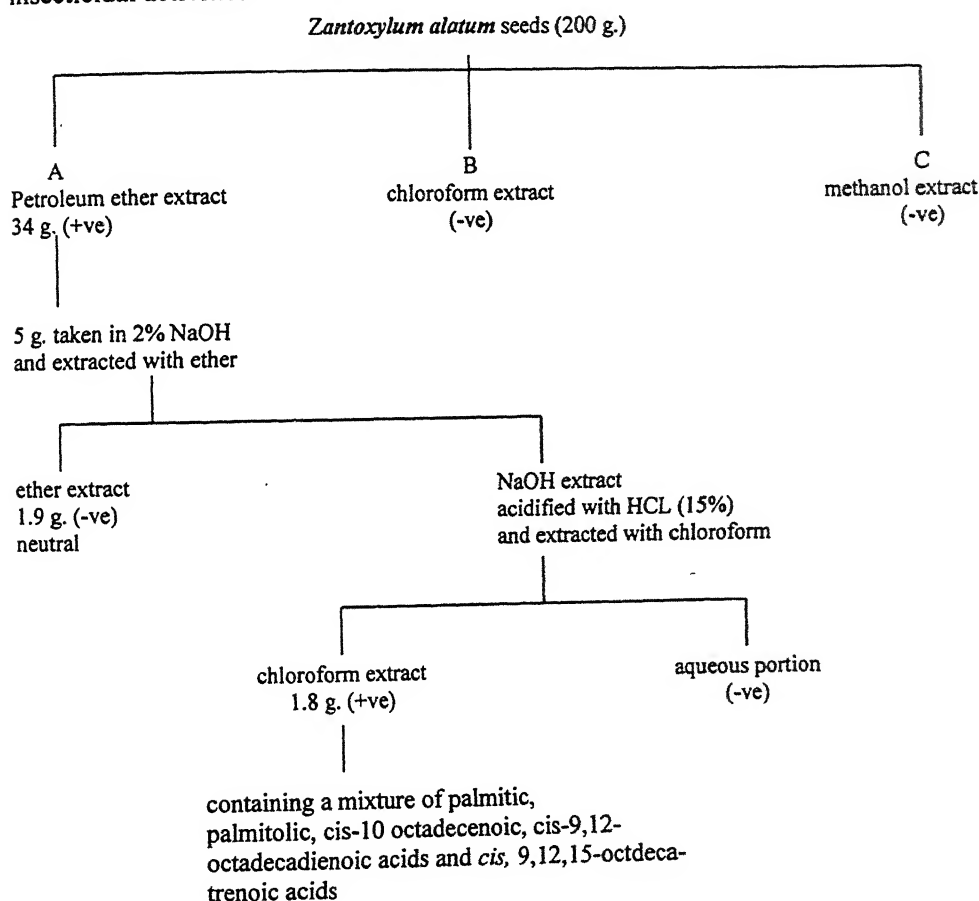


Fig. 1—Scheme for isolation of the active insecticidal principle of plant *Zanthoxylum alatum*.

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Bio-efficacy of *Clerodendrum siphonanthus* (Verbenaceae) against pulse beetle *Callosobruchus chinensis* (Coleoptera : Bruchidae)

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Abstract

The foliar part of *Clerodendrum siphonanthus* is dried at room temperature and extracted with petroleum ether, chloroform and methanol. Different concentrations are made from each crude extract and applied against *Callosobruchus chinensis* on filter paper. Observation is taken after 24 hours and its lethal concentration is calculated. Out of all the extracts, chloroform extract has shown highest mortality. The calculated LC₅₀ values of chloroform extract is 2.81 mg/ml, methanol extract is 2.95 mg/ml and petroleum ether is 3.46 mg/ml. Several fractions (200 ml each) are collected through column chromatography from the chloroform extract and out of these fractions, fraction VII and XI are found to be highly effective. Thin layer chromatography is done with these fractions using toluene : ethanol : chloroform (50:50:50). The R_f value of fraction VII (Green) is 0.89 and fraction XI (pale yellow) is 0.71.

(Key words : *Clerodendrum siphonanthus*/*Callosobruchus chinensis*/insecticide/bio-efficacy).

Introduction

Due to increased public awareness about immediate and long term hazardous effect of synthetic pesticides, botanical insecticides are presently getting increased attention because they are environmentally safe, bio-degradable and leave no toxic residues in soil, food and fodder¹. It has potentiality to preserve food grains like pulses which are heavily infested by stored grain pests during storage².

Clerodendrum siphonanthus R. BR (Verbenaceae), a tall erect shrub is locally known as Barangi in Hindi and Akalbih in Assamese³. the shrub has hollow stem and ashy grey, smooth bark. The flower is white in colour and fruit is 1.3 cm across, dark bluish green when ripe and seated on the enlarged red calyx. Its distribution is quite extensive. In India it is found from Madras (Chennai) in the South to Sikkim in the north and to Assam in the east. According to literature it is found from Tennessee in

USA to Sumatra in the far east^{4,5}. The medicinal value of *C. siphonenthus* has been reported by many authors^{6,7,8}. Some information is available on the isolation of a rice weevil feeding inhibitor from this plant. The two compounds uncinatone and pectoglinarigenin are reported from the fraction of petroleum ether leaf extract which shows inhibitory activity⁹. The two flavones, pectolinasigenin (I) and Cirsimartin (II) have been isolated from *C. siphonenthus* by column chromatography¹⁰.

The use of *C. siphonenthus* has generated a lot of scientific interest because of its reported insecticidal activity. Besides there are reports of many plants showing insecticidal activity against insects^{11,12,13}.

Some information is available about the chemical components of certain parts of *C. siphonenthus*⁵, for instance the bark of *C. siphonenthus*⁵ contains hexitol (mannitol) along with sorbitol⁵.

In the present experiment attempt has been made to assess the chemical which is responsible for showing insecticidal property against the stored product pest *Callosobruchus chinensis*.

Materials and Methods

Foliar extract of *Clerodendrum siphonenthus* :

C. siphonenthus leaves were collected from the field and dried at room temperature. The dried leaves were ground and extracted completely with petroleum ether (60-80 °C) for 4 days by cool extraction process. The same leaf materials were air dried and again extracted with chloroform for 4 days. Likewise the same material was dried and again extracted with methanol for 4 days. The extracts were then dried at room temperature in a vacuum desiccator.

Insect Rearing :

The pulse beetle *Callosobruchus chinensis* was collected locally and cultured in 1 kg container containing green gram at room temperature.

Bio-efficacy Testing of Extracts :

The petroleum ether (60-80 °C), chloroform and methanol extracts were diluted to different concentrations in ethanol (2.0, 2.25, 2.50, 2.75, 3.0, 3.50, 3.75, 4.00, 4.50 mg/ml) separately. Their 1 ml aliquots were sprayed on petriplate preloaded with Whatmann filter paper and then allowed to dry for 30 minutes at room temperature. In each plate 10 adult pulse beetles *Callosobruchus chinensis* were released after half

an hour and its activity and mortality were observed after 24 hours. Each test was done with three replications.

Fractionation of the crude extract and bioefficacy testing : The chloroform extract (2 gm) was run through column chromatography with the solvent petroleum ether and ethyl acetate in various ratios (10:0, 9:1, 8:2 and so on) and lastly with pure ethyl acetate. Thus several fractions (each of 200 ml) were collected and all the fractions were applied against *Callosobruchus chinensis*. Each fraction was diluted in ethanol and sprayed on filter paper and dried for half an hour at room temperature and the insects were slowly introduced in each petridish for assessing the active fractions.

Results and Discussion

The observation show that chloroform extract of *C. siphonenthus* leaf is more effective than methanol and petroleum ether extract. The insecticidal activity of *C. siphonenthus* and evaluation of LC_{50} is shown in Table 1, 2 and 3. The calculated lethal concentration. (LC_{50}) of petroleum ether extract is 3.46 mg/ml (Table 1), chloroform extract is 2.81 mg/ml (Table 2) and methanol extract is 2.95 mg/ml (Table 3). The chloroform extract was passed through column chromatography and several fractions are obtained. Of these, fraction VII was most effective (80% mortality), as also fraction XI (50% mortality) as compared to others (Table 4). The effective fractions were run in TLC using the solvent toluene : ethanol : chloroform (50:50:20). Two pure spots were observed. One single spot of green colour was observed in fraction VII having R_f value 0.89 and another single spot of pale yellow colour was observed in fraction number XI having R_f value 0.71.

From the present investigation it can be suggested that chloroform leaf extract is highly effective at low concentration (2.81 mg/ml) as compared to other extracts. The insecticidal application has been reported to show inhibitory effect on rice weevil also and the compounds isolated were uncinatone and pectolinasigenin from *C. siphonenthus*⁹. It is reported that the light petroleum ether (60-80 °C) extract on chromatography over silica gel affords a light yellow crystalline solid from the benzene ethyl acetate (9:1) elutes. The compound was identified as pectolinarigenin (I) (mp 200°C) which was earlier isolated from *C. phlomids* and *C. inerme*¹⁰. In the present experiment a yellow spot was obtained from fraction XI of the chloroform extract of leaf of *C. siphonenthus* when run in TLC using the solvent toluene : ethanol : chloroform (50:50:50). This fraction however had caused 50% mortality of *C. chinensis* (Table 4). Fraction VII which gave a single green spot on TLC was most effective causing 80% mortality in these beetles.

Table 1- Effect of Petroleum ether extract of *Clerodendrum siphonenthus* Leaf against *Callosobruchus chinensis* and evaluation of LC₅₀.

Conc. (mg/ml)	Total No. of Insects	Number of dead Insect	% of Mortality	Corrected Mortality	Logdose	Probit
2.00	30	2	6.6	7	0.301	3.52
2.25	30	5	16	16	0.352	4.01
2.50	30	9	30	30	0.397	4.48
2.75	30	12	40	40	0.439	4.75
3.00	30	14	46	46	0.477	4.90
3.50	30	16	53	53	0.544	5.08
3.75	30	18	60	60	0.570	5.25
4.00	30	22	73	73	0.602	5.61
4.50	30	26	86	86	0.650	6.08

The calculated LC₅₀ Value is 3.46 mg/ml.

Table 2- Effect of Chloroform extract of *Clerodendrum siphonenthus* Leaf against *Callosobruchus chinensis* and evaluation of LC₅₀.

Conc. (mg/ml)	Total No. of Insects	Number of dead Insect	% of Mortality	Corrected Mortality	Logdose	Probit
2.00	30	9	30	30	0.301	4.48
2.25	30	10	33.3	33	0.352	4.56
2.50	30	12	40	40	0.397	4.75
2.75	30	14	46.6	47	0.439	4.92
3.00	30	19	63.3	63	0.477	5.33
3.50	30	20	66.6	67	0.544	5.44
3.75	30	23	76.6	77	0.570	5.74
4.00	30	25	83.3	83	0.602	5.95
4.50	30	29	96.6	97	0.650	6.88

The calculated LC₅₀ Value is 2.81 mg/ml.

Table 3.—Effect of Mehanol extract of *Clerodendrum siphonenthus* Leaf against *Callosobruchus chinensis* and evaluation of LC₅₀.

Conc. (mg/ml)	Total No. of Insects	Number of dead Insect	% of Mortality	Corrected Mortality	Logdose	Probit
2.00	30	2	6.6	7	0.301	3.52
2.25	30	5	20	20	0.352	4.16
2.50	30	8	26.6	27	0.397	4.39
2.75	30	10	33.3	33	0.439	4.56
3.00	30	15	50	50	0.477	5
3.50	30	17	56.6	57	0.544	5.18
3.75	30	19	63.3	63	0.570	5.33
4.00	30	20	66.6	67	0.602	5.44
4.50	30	25	83.3	83	0.650	6.95

The calculated LC₅₀ Value is 2.95 mg/ml.

Table 4— Percentage of mortality of *C. chinensis* in some constituent fractions of chloroform extract of *Clerodendrum siphonenthus* Leaf.

Dose	Fraction	% of Moratility
0.5 mg	I	20
0.5 mg	V	30
0.5 mg	VI	30
0.5 mg	VII	80
0.5 mg	X	20
0.5 mg	XI	50

Thus it may be concluded that *C. siphonenthus*, is a locally available plant having great potentiality as a botanical insecticide.

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Propagation of *Coelogyne breviscapa* Lindle and *Coelogyne nervosa* A. Rich through asymbiotic seed germination

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Abstract

Seeds of *Coelogyne breviscapa* Lindl and *C. Nervosa* A. Rich were asymbiotically cultured on four different basal media. MS medium was effective in inducing seed germination in both the species. IAA enhanced seedling growth in *C. breviscapa* and NAA in *C. nervosa*. Among the cytokinins BAP significantly enhanced seedling growth compared to the control. Incorporation of coconut water and banana pulp in the medium did not affect the seedling growth significantly. Yeast extract (3mg/l) was found to be effective for seedling growth in *C. nervosa* and peptone (3mg/l) in *C. breviscapa*.

(**Key words** : *Coelogyne breviscapa*/*C. nervosa*/asymbiotic seed germination).

Introduction

Orchids constitute an order of royalty in the world of ornamental plants and they are of immense horticultural importance and also play a very useful role to balance the forest ecosystems¹. They occupy top position among all flowering plants valued for cut flower production and as potted plants, which fetch a very high price in the international market. The most common method of propagation of orchids is by division of pseudobulbs, cuttings and air layering. The *in vitro* techniques in orchid propagation have revolutionized orchid industry and resulted in the large number of uniform plants obtained within a short period for successful establishment of orchid industry.

In recent times, the wild orchids are also commercially exploited for horticultural trade. Although these species are still common, the development of an artificial means of seed propagation is needed to reduce collection pressures on wild population. Both tissue culture and seed propagation are options which could be used to rapidly increase plant numbers. Seed propagation may provide the best alternative, since it would conserve genetic variability. Hence the present study was attempted for mass

propagation and establishment of *Coelogyne nervosa*. endemic to western ghats and *Coelogyne breviscapa*.

Materials and Methods

The undehisced capsules (approximately 180-250 days old) of *Coelogyne nervosa*. A. Rich. and *Coelogyne breviscapa* Lindl. were collected from Naduvattam evergreen tropical forest at an altitude of 1800 meter above MSL and Mulakkadu evergreen tropical forest at an altitude of 1500 meter above MSL, Nilgiri district, respectively.

Four different basal media namely Knudson C², Murashige and Skoog³, Vacin and Went⁴ and B5⁵ were initially used for seed germination. The pH of all the media was adjusted to 5.8 prior to adding agar, 0.8% agar (Hi-media) was used as gelling agent. The prepared medium was dispensed in 25 × 30 mm borosil test tubes. The mouth of the test tubes were plugged with aluminium foil and autoclaved for 25 minute at 121 °C and 15 lb/in pressure. All the cultures were maintained under 12:12 h photoperiod (3000 lux light) at 25±2 °C and 70% relative humidity. After 10 days of inoculation, 20 samples were selected at random and observed under microscope and the germination percentage was calculated.

After 90 days the best medium was selected for further studies. The above medium was enriched with plant growth regulators, like NAA, IAA, BAP and KIN in different concentrations ranging between 0.2-1.0 mg/l and growth adjuvants like peptone, yeast extract (1-3 mg/l) and coconut water (5, 10 and 15%) individually to study their effects on the growth of the seedling. Three replicates were maintained for each treatment.

The well developed seedlings were removed from culture vessels and thoroughly washed with tap water to remove the adhering medium completely without causing damage to the roots. Then the plantlets were treated with the fungicide solution [Bevistin (carbendazim 50% WP)] at 5% concentration and transferred to plastic cups with the following media for hardening.

Vermiculite + brick pieces + charcoal + sphagnum moss in the ratio of 1:1:1:1.

and red soil + vermiculite + charcoal + sphagnum moss in the ratio 1:1:1:1.

The potting media were autoclaved and about 10 seedlings were placed in each cup. The hardening was maintained under low level polythene tunnels for well establishment.

Results

The seeds were aseptically removed and sown on four different basal media. Greening of the seeds were observed after four weeks in Knudson C medium followed by MS medium. The seed germination was delayed in Vacin and Went (fifth week) and B5 (sixth week) media. The germination percentage (60%) significantly decreased in B5 medium (Table-1a). During the germination the undifferentiated embryo emerged from testa and formed green protocorms (Fig 1). The protocorms developed first leaf and root during the fifth week in Knudson C medium followed by MS medium (sixth week). Though the protocorm differentiated first in Knudson C medium, the length of the first leaf, root and diameter of the protocorm in both the species were found to be more in MS medium. Hence, MS medium was selected further to study the effect of growth regulators and growth adjuvants on the development of seedlings.

Table 1a.— Effect of different basal medium on seed germination of *C. breviscapa* and *C. nervosa*.

S. No.	<i>Coelogyne breviscapa</i>			<i>Coelogyne nervosa</i>			
	Medium (Basal)	Time taken for germination	Time taken for protocorm formation	% of seed germination	Time taken for germination	Time taken for protocorm formation	% of seed germination
1.	MS	4 weeks	5 weeks	100	4 weeks	5 weeks	100
2.	KC	4 weeks	6 weeks	100	4 weeks	7 weeks	95
3.	V W	5 weeks	8 weeks	70	5 weeks	9 weeks	70
4.	B5	6 weeks	9 weeks	60	6 weeks	9 weeks	60

MS = Murashige and Skoog, KC = Knudson C

VW = Vacin and Went

Ninety day-old healthy protocorms obtained from MS medium were subcultured in the same medium containing different growth regulators individually at different concentrations. Among the auxins, IAA at 1mg/l increased the shoot and root length in *C. breviscapa* (Fig 2) whereas NAA at 1mg/l enhanced the shoot and root length in *C. nervosa*. The seedlings developed on MS medium supplemented with BAP (1mg/l) (Fig 3) were dark green in colour, and there was increase in the root and shoot length of both *C. breviscapa* and *C. nervosa*. Kinetin at the same concentration increase only the shoot length in both the species (Table 1b).

Table 1b—Effect of various growth regulators on seedling growth of *Coelogyne breviscapa* and *C. nervosa*.

Sl. No.	Medium growth regulator	Concentration (mg/l)	<i>Coelogyne breviscapa</i>		<i>Coelogyne nervosa</i>	
			Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)
			Mean±SE	Mean±SE	Mean±SE	Mean±SE
1.	MS+NAA	0.2	1.2±0.035	0.4±0.022	1.2±0.07	0.25±0.07
2.		0.5	1.3±0.035	0.45±0.022	1.3±0.054	0.3±0.015
3.		1.0	1.4±0.042	0.5±0.023	1.4±0.027	0.35±0.035
4.	MS+IAA	0.2	1.5±0.027	0.45±0.041	1.2±0.035	0.3±0.015
5.		0.5	1.6±0.035	0.50±0.022	1.3±0.016	0.35±0.035
6.		1.0	1.7±0.035	0.55±0.017	1.3±0.054	0.3±0.035
7.	MS+BAP	0.2	1.3±0.035	0.40±0.022	1.2±0.070	0.25±0.015
8.		0.5	1.3±0.035	0.35±0.032	1.3±1.054	0.2±0.063
9.		1.0	1.5±0.022	0.45±0.022	1.4±0.044	0.3±0.035
10.	MS+KN	0.2	1.3±0.035	0.35±0.032	1.1±0.070	0.2±0.063
11.		0.5	1.4±0.035	0.40±0.022	1.2±0.035	0.2±0.035
12.		1.0	1.8±0.035	0.40±0.022	1.3±0.016	0.25±0.015

Values are mean (n = 10) ± SE

The well developed protocorms obtained from MS medium were subcultured on the same medium containing various organic supplements like peptone, yeast extract and coconut water. Among the different growth adjuvants, peptone (3mg/l) (Fig 4) enhanced the root and shoot length in *C. breviscapa* whereas in *C. nervosa* yeast extract (3mg/l) was found to be suitable for subsequent seedling growth. Incorporation of coconut water, irrespective of concentration, did not affect the seedling growth significantly (Table 2). About 80% of the *in vitro* raised seedlings were established in the potting medium containing vermiculate + brick pieces + charcoal + sphagnum moss in the ratio 1:1:1:1.

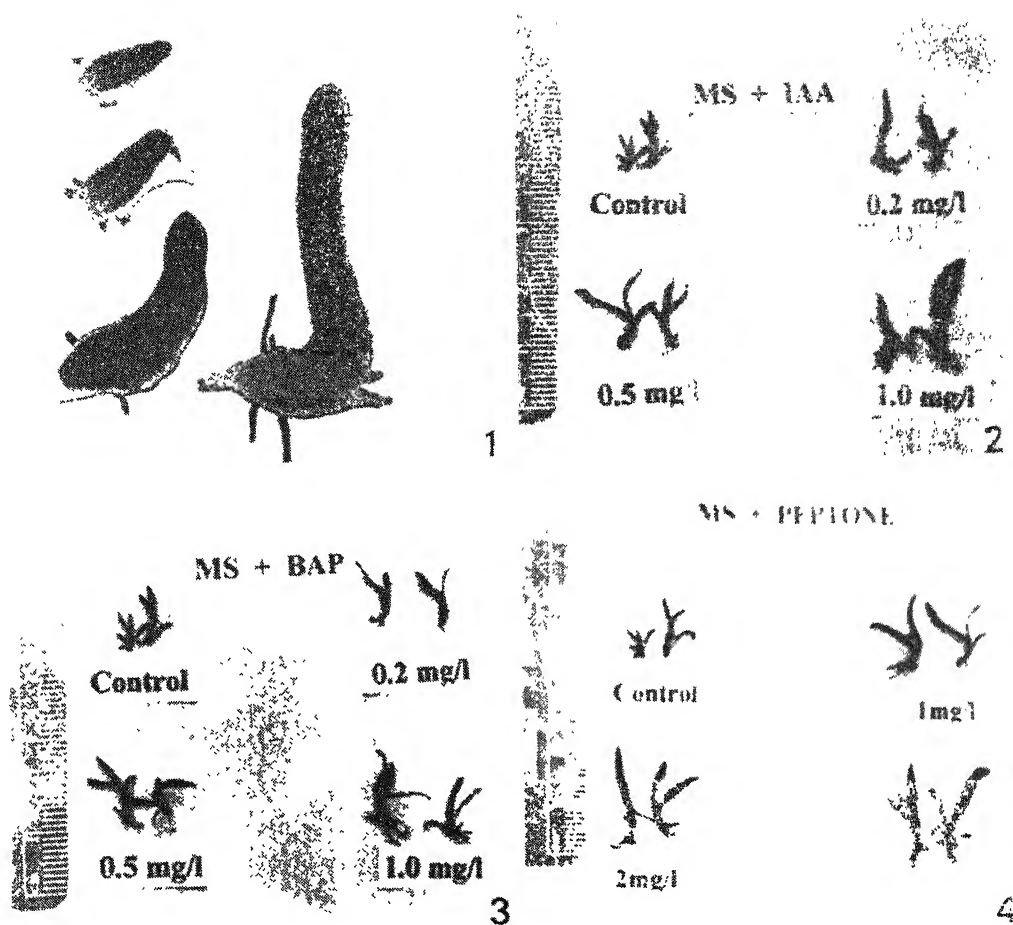


Fig. 1-4 *Coelogyne breviscapa*

1. Different stages in the development of protocorm.
2. Effect of IAA on seedling growth.
3. Same, BAP at different concentration.
4. Effect of Peptone on seedling growth.

Table 2.— Effect of growth adjuvants on seedling growth of *Coelogyne breviscapa* and *C. nervosa*

Sl. No.	Medium + growth adjuvants	Concentration	<i>Coelogyne breviscapa</i>		<i>Coelogyne nervosa</i>	
			Shoot length (cm) Mean \pm SE	Root length (cm) Mean \pm SE	Shoot length (cm) Mean \pm SE	Root length (cm) Mean \pm SE
1.	MS+peptone	1 mg/l	1.6 \pm 0.22	0.4 \pm 0.23	1.2 \pm 0.70	0.2 \pm 0.35
2.		2 mg/l	1.8 \pm 0.35	0.6 \pm 0.18	1.3 \pm 0.54	0.2 \pm 0.63
3.		3 mg/l	2.0 \pm 0.35	0.6 \pm 0.22	1.3 \pm 0.16	0.25 \pm 0.70
4.	MS+YE	1 mg/l	1.6 \pm 0.27	0.3 \pm 0.15	1.2 \pm 0.35	0.2 \pm 0.24
5.		2 mg/l	1.8 \pm 0.37	0.5 \pm 0.22	1.3 \pm 0.44	0.25 \pm 0.27
6.		3 mg/l	1.9 \pm 0.35	0.45 \pm 0.22	1.4 \pm 0.27	0.25 \pm 0.40
7.	MS+CW	5%	1.6 \pm 0.37	0.4 \pm 0.27	1.8 \pm 0.47	0.25 \pm 0.37
8.		10%	1.5 \pm 0.27	0.3 \pm 0.27	1.0 \pm 0.27	0.15 \pm 0.15
9.		15%	1.5 \pm 0.22	0.4 \pm 0.22	1.0 \pm 0.22	0.25 \pm 0.15

Values are mean (n = 10) \pm SE

Discussion

Tissue culture method for regeneration of orchid were introduced by several workers using various explants and culture media. Among the various explants, the shoot tip and axillary bud were commonly used. The seed germination in orchids in nature is an unique phenomenon and require fungal association. Germination is much more successful *in vitro*. In the present study among four media tried, the seeds of both *C. breviscapa* and *C. nervosa* though germinated early in Knudson C medium, MS medium was found to be more suitable for the development of protocorms (Table 1a).

The nutritional requirement varies with the genus, species and locality⁶. Several author suggested different nutrient media suitable at different stages of growth for various species. In the present study seeds of both *C. breviscapa* and *C. nervosa* germinated and developed seedlings on all the media used. MS medium proved to be better for the development of protocorms.

Addition of growth regulators and organic supplements is needed in some species for the enhancement of seed germination and seedling growth. In majority of the cases auxins enhanced germination and seedling growth⁷. In the present investigation, NAA (1mg/l) was effective in promoting seedling growth in *C. nervosa* and IAA *C. breviscapa*. The effectiveness of these auxins for growth of orchid seedlings has been reported by many investigators^{8,9} BAP and Kinetin had more or less the same effect on the development of seedling in both the species. Kinetin (1-10mg/l) retarded seed germination and seedling growth in *Coeloglossum viride* and *Plantanthera bifolia*¹⁰.

Among the organic supplements employed, peptone at 3 mg/l in the medium enhanced the shoot length in *C. breviscapa* and yeast extract at 3 mg/l in *C. nervosa*. The shoot development was significantly affected by coconut water as reported in *Dendrobium chrysanthum* and *Paphiopedilum spicerianum*¹¹. The *in vitro* seedlings were successfully established in potting medium. Further studies will be carried out to establish the *in vitro* raised seedlings in their original habitat.

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Mycoflora spectrum during storage and its effect on seed viability of soybean [*Glycine max* (L.) Merrill] seeds under ambient conditions

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Abstract

Soybean seed of JS-80-21 and Pusa-16 varieties were sampled and treated with mancozeb, thiram, numbecidine and bleaching powder and stored in two types of containers under ambient storage conditions. The percent occurrence of thermophilic/thermotolerant flora was 29.8% as against 70.2% of mesophilic flora, irrespective of treatments. Amongst the two storage containers, polylined bag harboured low incidence of mycoflora (37.6%) as compared to cloth bag packaging (62.4%). Amongst seed treatments, mancozeb (78.6%) and thiram (65.1%) seed dressings controlled seed mycoflora more effectively than treatments with nimbecidine (10.1%) and bleaching powder (13.0%). With advancing storage period, the field fungi (*Cladosporium cladosporioides*, *Alternaria alternata*, *Acremonium* sp., *Fusarium moniliforme*, *F. semitectum*, *Curvularia lunata* and mycelia sterilia) were replaced with storage fungi (species of *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Chaetomium*, *Epicoccum* and *Trichothecium*). The reasons for appearance and non-appearance of seed mycoflora during storage can be attributed to the variations in temperature and RH of seed godown. A highly significant negative correlation ($r = -0.8718$) between seed viability and seed mycoflora suggest seed mycoflora to be an important factor responsible for seed deterioration during storage under ambient conditions.

(**Key words** : soybean/seed mycoflora/storage/seed viability/seed treatment).

Introduction

The role of seed mycoflora in seed spoilage is being increasingly recognized during recent years^{1,2}. The loss due to fungi can be especially high under tropical and sub-tropical conditions where high humidity and temperature coupled with poor storage conditions exist and the mechanical injuries caused to the seed during harvesting facilitate easy entry of fungal pathogens during storage. The field and storage fungi virtually classified on the basis of the stage at which invasion and growth of fungi occur in/on the seed, play certain role in the deterioration of stored

products³. Temperature variations in the seed godown also play an important role in the development of the storage fungi. Broadly speaking, amongst the seed borne fungi, the primary invaders during storage may be either mesophilic or thermophilic. Thermophilic fungi may be present as superficial contaminants of grain before harvest but can invade grain kernels when spontaneous heating occur in storage, and therefore can be regarded as storage fungi. Proper storage and application of safe chemicals as post-harvest treatment can considerably reduce the losses in storage. The available literature indicates that the work done on storage losses in agricultural products is mainly confined towards understanding the ecology of seed mycoflora of any one group of microorganisms. The present work was initiated to study the mycoflora on soybean seed at different temperatures, their persistence in different treatments and their effect on seed viability during storage under ambient conditions.

Materials and Methods

The processed seed lots of two varieties of soybean- JS-80-21 and Pusa-16, harvested during December 1995, were sampled and treated with mancozeb 75% WP, thiram 75% DS and bleaching powder each @ 2.5/kg seed and with nimbecidine (azadirachtin 0.03 % EC)@ 2.5 ml/kg seed as dry dressing. One sample was left untreated, which served as control. One half of these samples were stored in cloth bag and the other half in polythene bag of 400 gauge inside a cloth bag, henceforth referred to as polylined bag. These subsamples were kept inside a seed godown at IARI, Regional Station, Karnal under ambient storage condition.

Storage conditions of seed godown :

The minimum and maximum temperatures and relative humidity of the seed godown was recorded daily using Heal's thermometer and Huger's Hygrometer respectively, from February 1996 to Feb., 1997. The minimum temperature of the seed godown varied from 13.4 °C to 37.5 °C during the same months. The relative humidity of the seed godown varied from 51.9 per cent in April 1996 to 75.4 per cent in January 1997 (Fig. 1).

Seed mycoflora :

The fungal flora associated with seeds in different treatments was studied following blotter test method. The seeded plates were incubated for 5 days at 45 °C to isolate thermophilic fungi, and at 28 °C for 7 days to isolate mesophilic fungi. Percent occurrence of fungi was calculated as:

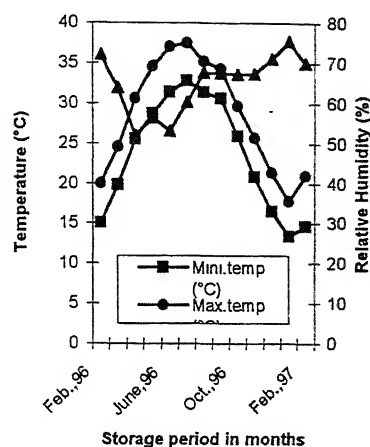


Fig. 1—Temperature and relative humidity of seed godown during storage.

$$\frac{\text{Total number of seeds in which a particular fungus occurred} \times 100}{\text{Total number of seeds incubated}}$$

Average percent seed mycoflora control was calculated as :

$$\frac{\text{Average incidence of seed mycoflora in control treatment} - \text{Average incidence of seed mycoflora in fungicidal treatment}}{\text{Average incidence of seed mycoflora in control treatment}}$$

Average incidence of seed mycoflora in control treatment

The mycoflora found associated with soybean seeds in different treatments has been grouped into four categories—

Category I Seeds of JS-80-21 variety, stored in cloth bag packaging,

Category II Seeds of JS-80-21 variety, stored in polylined bag packaging,

Category III Seeds Pusa-16 variety, stored in cloth bag packaging,

Category IV Seeds Pusa-16 variety, stored in polylined bag packaging.

Results and Discussion

Ten species were recorded on the seeds in soybean 'cv' JS-80-21, under thermophilic/thermotolerant group and 19 species were recorded under mesophilic group (Table 1). In 'cv' P-16, 12 and 17 spp. were recorded under thermophilic/thermotolerant and mesophilic groups respectively, irrespective of treatments. In all, five species of *Aspergillus* were found associated with seeds of P-16 variety, whereas *A. candidus* was found to be absent in JS-80-21 variety. *Trichoderma* sp. and mycelia sterilia were missing on the seeds of P-16 variety. *Trichothecium roseum* was recorded on the seeds of both the varieties stored in cloth bag packing only. The percent occurrence of *Cladosporium cladosporioides* as mesophile was maximum and it decreased with increasing storage duration whereas an actinomycete and *Aspergillus fumigatus* was recorded as thermophile at the time of storage. *A. flavus* and *Fusarium moniliforme* were also recorded as thermophile whereas *F. semitectum* and *T. roseum* occurred as mesophiles. The percent occurrence of thermophiles and mesophiles on the seeds of JS-80-21 variety was 47.3% as against 52.6% found associated with seeds of P-16 variety (Fig. 2). The percent occurrence of thermophilic/thermotolerant flora was 29.7% as against 70.2% of mesophilic flora, irrespective of treatments. The incidence of fungal species associated with seeds was low in polylined bag packaging (37.6%) as compared to 62.3% in cloth bag packaging. Also, 33.5% and 28.9% mycoflora was recorded in category I and III as against 13.8% and 23.8% in categories II and IV, respectively (Fig. 3). Further, 7.8% and 9.9% of thermophilic/thermotolerant species were recorded in categories I and III as against 4.9% and 7.3% in categories II and IV respectively. 25.7% and 19.0% of mesophilic flora was recorded in categories I and III as against 8.9 and 16.5% in categories II and IV, respectively. Though the incidence of thermophilous flora associated with soybean seeds was observed to be lower than mesophilic mycoflora but their importance during storage resulting in seed deterioration needs more exploration.

The rapid increase in the fungal population in cloth bag packaging during the initial phase of storage can evidently be attributed to the significant rise in RH during the rainy season coupled with increasingly favourable temperature (Fig. 1). The moisture content of seed varied from 5.5 to 11.1 per cent in different treatments. Arafa and co-workers⁴ also observed that fungal invasion was greatest after four months of storage coinciding with highest seed moisture content, and on prolonged storage both the temperature and RH registered a comparative decline and hence the total population did not show any significant rise. Low fungal populations recorded on seed stored in polylined bag as against those stored in cloth bag, may be due to the closed atmosphere of the polylined bag, which does not allow the environmental fluctuations

to affect the seed and seed mycoflora. It may then gradually become rich in the CO₂ contents and may inhibit the fungal growth and population. Thus, RH and temperature of the seed godown might be responsible for seed deterioration during storage under ambient condition. Zagrebenyer and Bern² were also of the view that storage fungi were the major factor causing soybean seed deterioration.

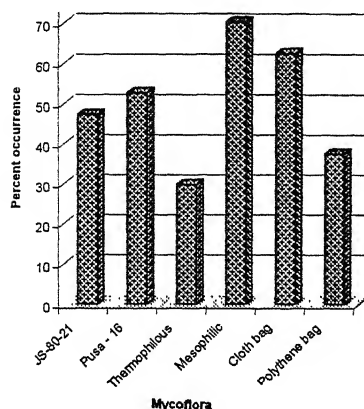


Fig. 2—Percent occurrence of mycoflora amongst varieties (JS-80-21 & P-16), groups (thermophilous & mesophilic) and storage containers (cloth & polylined bag).

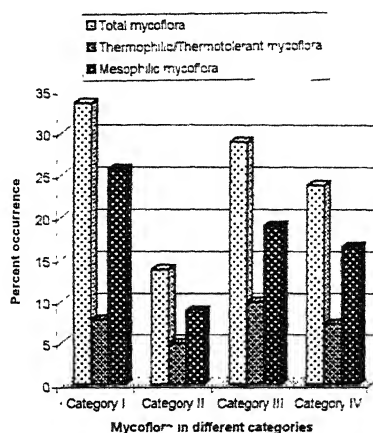


Fig. 3—Occurrence of total mycoflora, thermo-philic/ thermotolerant and mesophilic mycoflora in different treatments.

The effect of seed dressings was similar in both the varieties of soybean stored in both the containers, though percent occurrence of storage fungi especially, *A. flavus*, *A. niger*, *A. fumigatus*, *A. ochraceus* and *Rhizopus stolonifer*, were higher in categories I and III as against categories II and IV (Table 1). Kushwaha and Raut⁵ had reported that the seeds treated with thiram and stored in polylined bag suppressed most of the fungi. Our studies also showed that seed treatment with maniozeb and thiram reduced the incidence of fungi on seeds as against other seed treatments. Only 6.4 and 10 per cent of the total seed mycoflora was present associated with mancozeb and thiram treated seeds respectively, as against 26.9% in nimbecidine and 26.1% in bleaching powder treatments, which were almost at par with control (30.0%). On calculating the average percent seed mycoflora control, mancozeb (78.6%) was most effective during storage followed by thiram (65.1%), bleaching powder (13.1%) and nimbecidine (10.1%), indicating the ineffectivity of nimbecidine and bleaching powder in controlling seed mycoflora during storage as against the fungicides (Fig. 4). Asalmol and Zade⁶ also observed that pre-storage seed treatment helps to improve the shelf life of seeds and checks seed mycoflora during storage.

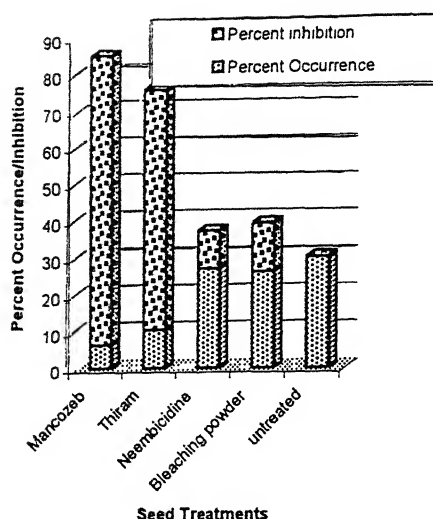


Fig. 4—Influence of seed dressings on percent occurrence and inhibition of seed mycoflora.

Persistence of mycoflora during storage :

The persistence of fungi/actinomycete on the stored seeds differed in different categories. The actinomycete was consistently present during storage on the seeds as thermophile. Amongst the fungi, *Aspergillus fumigatus* was most consistently found associated with the seeds of all the categories as thermophile. Species of *Penicillium*, *Rhizopus* and *Aspergillus* except *A. flavus* appeared later during the storage period and were absent initially at the time of storage. *A. flavus* was present during storage period. *Mucor* was initially absent in categories III and IV and irregular in appearance in categories I and II. Most of the mesophilic fungi like *Fusarium semitectum*, *Alternaria alternata*, *Curvularia lunata*, *Trichothecium roseum*, *Chaetomium spinosum*, *Epicoccum nigrum*, *Acremonium* sp. (= *Cephalosporium* sp.) and *Trichoderma* sp. were present irregularly on the seeds in different categories. The storage fungi like species of *Chaetomium*, *Epicoccum* and *Trichothecium* also appeared at a later stage during storage. The incidence of *Cladosporium cladosporioides*, *Alternaria alternata* and *Acremonium* sp. declined with increase in the storage duration, and thus they behaved as field fungi. Nakka⁷ also observed a decline in the incidence of *Cladosporium* and *Alternaria* and increase in the incidence of *Aspergillus* and *Penicillium* with advancing storage period. The variations in the storage conditions i.e., temperatures and relative humidity of the uncontrolled seed godown, may be the reasons attributed for appearance and non-appearance of fungi during storage.

Influence of seed mycoflora on seed viability :

With increase in the storage duration there was increase in the incidence of seed mycoflora but decrease in seed viability. The reason for the loss of seed viability are not fully understood but seed ageing and physiological changes in seed during storage are usually ascribed as possible factors responsible for seed deterioration. Though Periera *et al*⁸. opined that the fungi were not the main cause of low seed quality during storage, but our studies have indicated that seed mycoflora has high negative correlation ($r = -0.8718$) with seed viability and a positive correlation with seed moisture ($r = 0.35$) during storage (Table 2). This suggested that seed moisture influence seed mycoflora which might be responsible for bringing about certain physiological changes in the seed, leading to loss in seed quality during storage. Vaidehi¹ showed that the storage fungi lowered the quality of maize grains due to the biochemical changes brought about by them. Seed treatment with mancozeb/thiram and storage in polylined bag retained seed viability of soybean 'cvs' P-16 and JS-80-21 for 15 and 13 months after seed harvest respectively, as against 9 months in untreated seeds stored in cloth bags. These fungicides may be acting as protective agents against seed deterioration due to fungal invasion and physiological ageing as a result of which the seed viability was maintained for a comparatively longer period under ambient conditions.

Table 2.— Influence of seed mycoflora on seed viability and seed moisture during storage.

Storage period (in months after seed treatment)	Seed Germination* (%)	Seed Mycoflora* (%)	Seed Moisture* (%)
0	92.0	1.05	7.8
2	86.5	2.4	5.9
4	92.8	3.4	6.6
6	85.3	2.7	9.9
8	50.8	5.9	8.6
10	44.3	4.6	8.9
12	38.3	5.0	8.8
Correlation coefficient (r)	-0.872		0.35

*Average of 20 treatments

Thus, it is inferred from the present studies that both seed treatments and storage containers influence the percent mycoflora associated with soybean seeds during storage for 15 months after seed harvest under ambient conditions. In general, there was an increase in the number of the fungal colonies and decrease in seed viability irrespective of treatments and containers with the advancement in storage period. Of the two storage containers, polylined bag was found superior since the seed stored in it harboured less number of fungal colonies as compared to seed stored in cloth bags and seed treatment with fungicides like mancozeb/thiram prior to storage accorded protection to the seeds during storage and maintained seed viability for longer duration ambient conditions.

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Proline accumulation and lipid peroxidation in wheat seedlings exposed to cadmium toxicity

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Abstract

Imposition of increasing concentrations of cadmium in the form of cadmium chloride on wheat seedlings drastically decreased root growth. An uniform increase in the accumulation of proline, an osmoprotectant was noticed in both leaf and root tissues on 12th day of study. Roots showed more proline accumulation than leaves. Lipid peroxidation measured in terms of malondialdehyde content (MDA) and membrane injury index (%) increased with increasing metal concentration in both leaf and root tissues.

(Key words : proline/lipid peroxidation/cadmium/*Triticum aestivum*.)

Introduction

Cadmium is known to affect seed germination, seedling growth and metabolic process in plants. It induces oxidative stress producing reactive oxygen species like O₂, H₂O₂, OH etc. causing high degree of membrane lipid peroxidation and other oxidative injuries¹⁻⁵. The present experiment was carried out to study some of these aspects of Cd toxicity in wheat, a widely cultivated cereal.

Materials and Methods

Dry graded uniform wheat (*Triticum aestivum* L. cv. Sonalika) seeds were taken and surface sterilized with 0.1% HgCl₂ for 5-8 minutes and were thoroughly washed and rinsed with distilled water. Seeds were germinated in different petriplates containing moistened whatman No. 1 filter paper and were kept in dark for 2 days at 25 ± 2 °C. On second day germinated seeds were transferred to plastic glasses containing half strength Hoagland nutrient solution and were kept in growth chamber under continuous white light provided by filtered, cool white fluorescent tubes (Philips 36 W TLD) with aphoton flux density of 52 µ molm⁻²s⁻¹. On seventh day Cd stress treatments were imposed in the form of cadmium chloride solutions (0,0.001,

0.01, 0.1, 1.0 mM). The effect of stress treatments were studied on 12 day old wheat seedling. Proline estimation of both the root and leaf was done as per the method described elsewhere⁶. Homogenates of roots and leaves were prepared with 5% TCA and were taken for extraction and estimation of malondialdehyde (MDA)⁷. For measuring membrane injury index, 200 mg of root and leaf tissues were placed in 15ml deionised water and incubated for 24 h at 25 °C. The electrical conductivity of the medium was measured after 24 h. The tissue with leachate was then autoclaved at 1 kg cm⁻² pressure. The electrical conductivity of the medium was again measured at room temperature. The membrane injury index was calculated as per the method described elsewhere⁸.

Results and Discussion

Increasing concentrations of cadmium decreased the root length growth in 12 day old seedlings as compared to control. However, maximum decrease occurred at 10 mM Cd concentration. Similar inhibition in root elongation has been reported earlier in different plants suggesting the sensitiveness in terms of growth to the metal which seems to be due to a decrease in root absorption area, inactivation or non-induction of key defence machinery⁹. Proline accumulation showed an uniform trend in both leaf and root. However, root cells showed more accumulation of free proline at higher metal concentration. Accumulation of free proline in response to heavy metal exposure seems to be wide spread among plants. The possible reason of accumulation of this osmoprotectant proline in plants under cadmium toxicity is that it is induced by a Cd imposed decrease of the plant water potential and the functional significance would be in the contribution of proline to water balance maintenance^{1,3,10,11}. It is also attributed for scavenging of hydroxyl radicals since heavy metal exposure is known to induce oxy-free radical production and lipid peroxidation¹²⁻¹³.

A drastic increase in lipid peroxidation as evident by malondialdehyde (MDA) accumulation and membrane injury index (%) was recorded with the increase in metal concentrations in tissues of 12d old wheat seedlings. Similar reports are available for different plants^{5,14}. Increase in the level of MDA suggests generations of hydroxyl radicals either by Haber-Weiss or Fenton type reactions¹³. The reason for membrane injury in root tissue than leaf may be due to the heavy metal reaching the shoot is usually lower than the amount present in roots or because of the formation of phytochelatin and binding of Cd to root cell walls¹⁵⁻¹⁶.

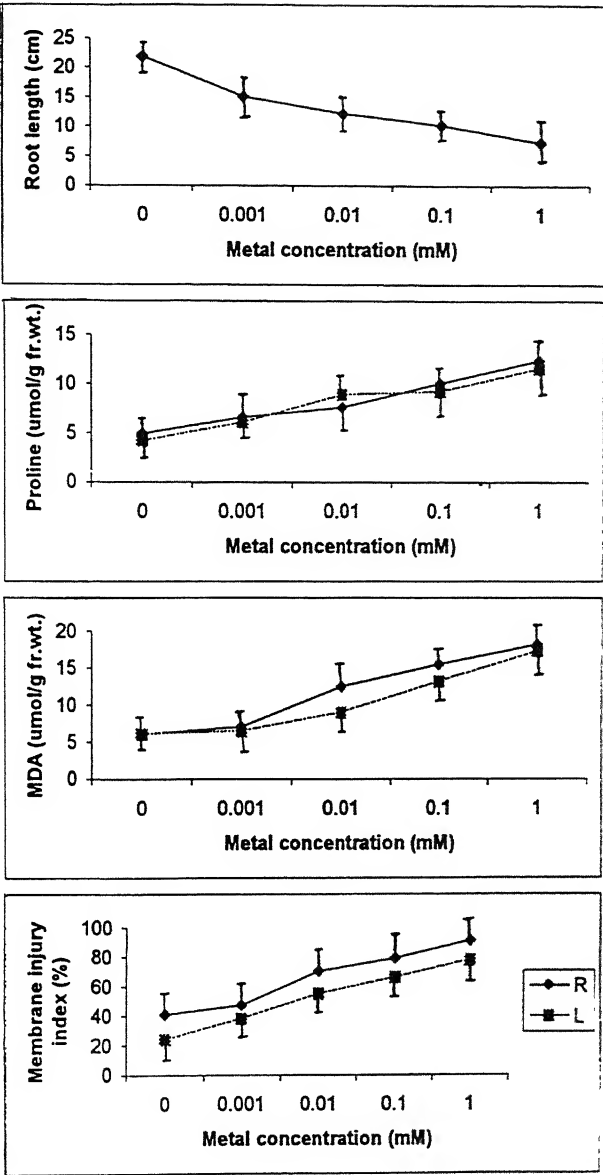


Fig 1.—Changes in root elongation, proline accumulation, malondialdehyde (MDA) content and membrane injury index (%) parameters in root and leaf cells of 12-d old wheat seedlings under cadmium toxicity. Data presented are mean \pm SEM.

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Studies on the biological treatment of digested distillery spent wash effluent using mutant strain of *Phanerochaete chrysosporium*

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Abstract

A new mutant strain, *Phanerochaete chrysosporium* GPS-V, showing high decolourization and COD removal capability on digested distillery spent wash effluent (40% v/v) in basal medium was induced from *Phanerochaete chrysosporium* isolated from lagoon soil by enrichment culture technique. In submerged cultures with basal medium containing 40% (w/v) digested spent wash supplemented with additional carbon source, glucose 4% (w/v) and other nutrients, the maximum decolourization of 84% and COD reduction of 91% was achieved at the optimum performance parameters.

(**Key words** : decolourization/digested spent wash/distillery effluent/ *P. chrysosporium*).

Introduction

Anaerobically digested cane molasses spent wash is a dark brown coloured effluent with high chemical oxygen demand (COD), high pollution potential and is bioremediation resistant. The colour is mainly due to the melanoidins, brown polymers which are formed by the Malliard amino-carbonyl reaction¹ and partly due to lignin which are not toxic as such. When these coloured effluents enter rivers or any other surface water system they upset biological activity causing detriment to the aquatic life^{2,3}. Disposal of this recalcitrant effluent on land further causes a reduction in soil alkalinity, manganese availability and effecting the ground water system because of the leaching from the soil^{4,5}. The treatment of spent wash through physicochemical process is cost intensive and the sludge disposal makes the process further uneconomical⁶. Biological treatment methods under natural conditions such as activated sludge or anaerobic lagooning are intricate⁷ and in general, are une-

conomical because of high operational cost and requirement of large land area. Hence, researchers in past have tried bioremediation using various melanoidin and lignin degrading microbes. Microbial decolourization of melanoidin has been detected in fungi. Ohmomo⁸ reported a strain of *Aspergillus sp.* showing high decolourization activity by the adsorption phenomena of melanoidin to fungal mycelia. Several other white rot fungi decolourizing melanoidin degradation were also reported^{11,12}

In the present communication we report the isolation of a mutant strain of *Phanerochaete chrysosporium*, capable of both bioremediation and decolourization of digested distillery spent wash at higher concentrations.

Materials and Methods

Materials:

The anaerobically digested spent wash was collected from local distillery of Dehradun, India. The effluent was stored in dark at low temperature under refrigeration before being used as substrate for biological treatment.

Organism :

The fungus *Phanerochaete chrysosporium*, was isolated from the lagoon soil by enrichment with digested spent wash¹³. It was maintained on malt extract agar slants, stored at 4 °C and renewed every month.

Mutagenesis and screening :

The mutagenesis of *Phanerochaete chrysosporium* was done according to the standard method¹⁴. The effluent decolourizing capability of the mutagenised colony was done by measuring the diameter of the halozone. The digested spent wash 10% (v/v) was used as substrate for examining decolourization activity on agar plates. The mineral base medium consist of 2% glucose, 0.5% yeast extract, 0.1% KH₂PO₄, 0.05%, MgSO₄.7H₂O and 2% agar at pH 6.0.

Preparation of the inoculum :

Spore suspension was prepared by adding 10 ml of sterile distilled water to the five day old slants of culture. 5 ml of this culture suspension was transferred to 50 ml

of potato dextrose broth (PDB) and incubated for 72 h. at 30 °C in shake flasks. The growth obtained was used as an inoculum for the microbial treatment of the digested spent wash.

Shake flask decolourization experiments :

All the studies for decolourization of digested spent wash were carried out in a basal medial composed of 10%-80% (v/v) digested spent wash containing 2% glucose, 0.5 NH₄NO₃, 0.1% KH₂PO₄ and 0.05% MgSO₄.7H₂O. The pH of the medium was adjusted to 6.0 unless otherwise specified. The experiment was carried out by dispensing 100 ml of basal medium of different concentration of digested spent wash in 250 ml conical flasks, incubated at 30 °C on a rotatory shaker at 150 rpm. At appropriate time flasks were withdrawn and contents were analysed for decolourization, COD, total sugar and total solid contents. The observations reported were measured after 8 days of incubation.

Analytical methods :

The decolourization of digested spent wash was measured as a decrease in optical density at 465 nm of diluted spent wash medium and expressed as percentage decrease in absorbance. Analyses to determine the specific gravity, total reducing sugar, total sugar, total suspended solids, carbon, nitrogen, phosphorus, COD, electrical conductivity etc, for digested effluent were those described in APHA (1989)¹⁵.

Results

The primary treatment of distillery effluent with anaerobic bacteria, recovering fuel gases and removing 80-85% BOD/COD is a most attractive proposal for treatment. The remaining 15-20% BOD/COD is very hard to remove. This is attributable to change in the ratio of biodegradable to non-biodegradable components, end product inhibition or due to the highly complex biorefractory compounds melanoidin which is not easily degraded by the microorganisms although it is highly distributed in nature. The chemical components digested spent wash are shown in Table 1.

Isolation of a high decolourizing potential mutant strain of *P. chrysosporium*:

Fig. 1 shows the population distribution of *P. chrysosporium* with respect to decolourization potential in terms of diameters of halozones. The enrichment culture

method generated a high decolourization activity potential strain giving halozone values (diameter) of 8-10 mm. On further mutanization by UV in the presence of enriched basal medium resulting in a mutant designated as *P. chrysosporium* GPS-V which showed higher halozone diameter values than the previously reported isolated strain¹³. The initial activity of fungal strain was clearly evident with the maximum ability of decolourization of 90% and COD reduction of 97% at 1% (v/v) of digested distillery spent wash in basal medium in shake flask experiments. This potential of fungal strain was substantially enhanced by the consecutive optimization of the process.

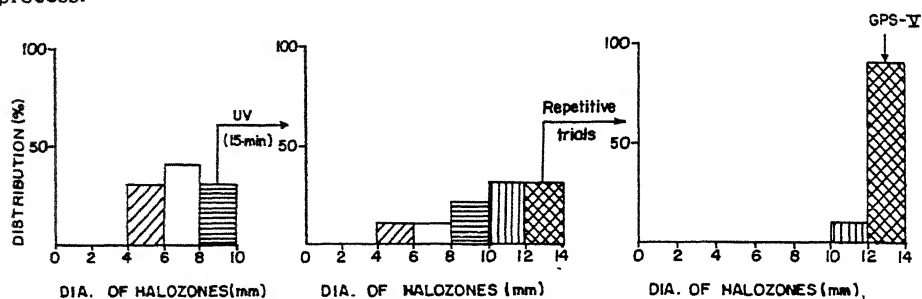


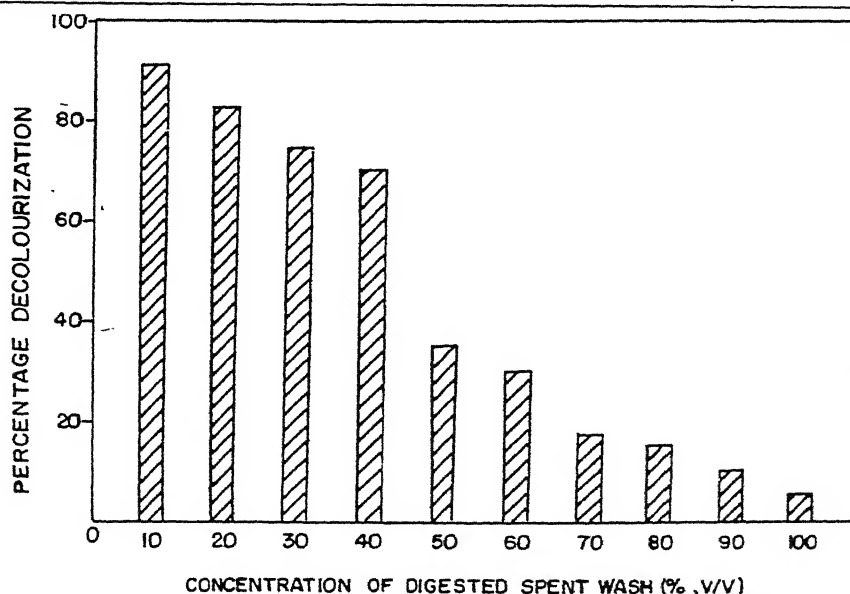
Fig. 1—Selection of mutant strain of *Phanerochaete chrysosporium* for high decolourization activity of digested distillery spent wash after UV mutanization.

Effect of different concentrations of digested spent wash on declourization activity :

The effect of different concentrations of digested spent wash in basal media on decolourization and COD reduction by the fungal culture is shown in Fig. 2. Increased concentration of effluent in basal media reduce both percentage decolourization and COD removal. The potential of the fungal strain was clearly evident upto 40% (v/v) digested spent wash in basal media. Above this concentration level the fungal activity declined rapidly. The inhibitory effect of increasing concentration is likely to be due to the presence of the inhibitory compounds such as phenolics, gallic acid and vanillic acid and additionally due to respective increase in osmotic potential of fermentation medium which hinders nutrient uptake and release of metabolites thus effecting mycelial growth. This shows that substantial dilution is required for spent wash before effective decolorization and COD reduction can be achieved.

Table 1- Chemical composition of Raw and Digested spent wash

Parameters	Raw spent wash	Digested spent was
pH	4.60	7.66
Density	1.14	1.03
Total Solids (g/l)	107.10	32.40
Volatile (g/l)	41.40	16.92
Dissolved solids (g/l)	24.40	7.69
Tatla carbon (g/l)	46.00	8.30
Total nitrogen (g/l)	3.63	1.23
Total phosphorous (g/l)	0.09	0.09
Total sugar (g/l)	29.65	14.30
COD (mg/l)	1,22,000.00	28,600.00
BOD (mg/l)	16,000.00	1,800.00

Fig. 2-Percentage decolourization by *Phanerochaete chrysosporium* at different concentrations of digested spent wash in basal medium.**Effect of different carbon and nitrogen source on decolourization activity :**

The medium containing 40% (v/v) digested spent wash in basal medium was supplemented with different carbon sources in the form of sugars namely glucose,

sucrose, maltose, fructose, mannose, galactose, mannitol, xylose. It was observed that glucose, sucrose and maltose induced the maximum growth and decolourization in 8 days of incubation. Further, the optimal concentration of glucose was determined. It was found that maximum decolourization and COD reduction achieved under the optimum supplementation of glucose 4% (w/v) (Table 2). This shows that digested wash contained little, if any, readily available carbon inspite of its high total sugar content. Therefore, it showed a proportional increase in percentage decolourization upto 4% (w/v) glucose. Increasing glucose concentration further did not affect the decolourization. The respective increase in decolourization with uptake of glucose reflects that the components of spent wash does not serve directly as nutrients for fungal growth, instead a secondary metabolite is being released, degrading the melanoidins extracellularly.

Table 2– Effect of added glucose and nitrogen sources on digested spent wash decolourization after 8 days of incubation.

Source (%)	Decolourization (%)	COD removal (%)	Total solids reduction (%)	Total sugar reduction (%)
Glucose (%)				
1	43.23	46.60	48.60	49.96
2	77.93	84.04	87.28	88.96
3	80.53	86.60	90.53	91.66
4	83.88	90.41	94.29	95.66
5	84.00	90.77	94.31	95.85
6	83.50	90.00	93.78	95.04
NH₄NO₃ (mM/100 ml)				
2.5	74.69	80.51	87.30	90.44
5.0	78.15	84.23	91.05	93.23
7.5	80.13	86.50	93.65	96.20
10.0	80.05	87.25	94.31	96.70
12.5	80.24	87.00	94.04	96.00

Supplementation with various organic & inorganic nitrogen sources also exhibited the marked impact on COD and decolourization activity (Table 2). Among the various inorganic nitrogen sources like nitrate, sulphate and chlorides of ammonium, tested, NH₄NO₃ was found to be very effective. Although the maximum activity of decolourization and COD reduction was achieved utilizing yeast extract and peptone. But for the technoeconomic feasibility and further scale-up of the process the use of NH₄NO₃ as the supplemented nitrogen source was better option for the

decolourization and COD reduction in spent wash. Further the optimal concentration of nitrogen source was determined and it was observed that at 0.3% (w/v), NH_4NO_3 i.e. 7.5 mM/100 ml induced the maximum decolourization and COD reduction.

Time Course of decolourization activity :

Under optimal performance parameters, supplementation of glucose, 4% (w/v), NH_4NO_3 , 0.3% (w/v), optimal pH 5.0 and temperature 35 °C, the decolourization and COD reduction was observed as a function of time. The results showed the increase in the biomass accumulation with corresponding decrease in the total sugar concentration between 0 to 4 days of incubation. The percentage decolourization and COD reduction in the same period shows a slow trend. The rate of microbial activity of decolourization enhanced between 4 to 8 days of incubation. Lastly, the rate of decolourization slowed down between 8 to 12 days of incubation (Fig. 3). In addition to this, supplemented readily available carbon source depleted within 2 days of incubation. This shows that the glucose supplementation was necessary to initiate the growth of fungal culture.

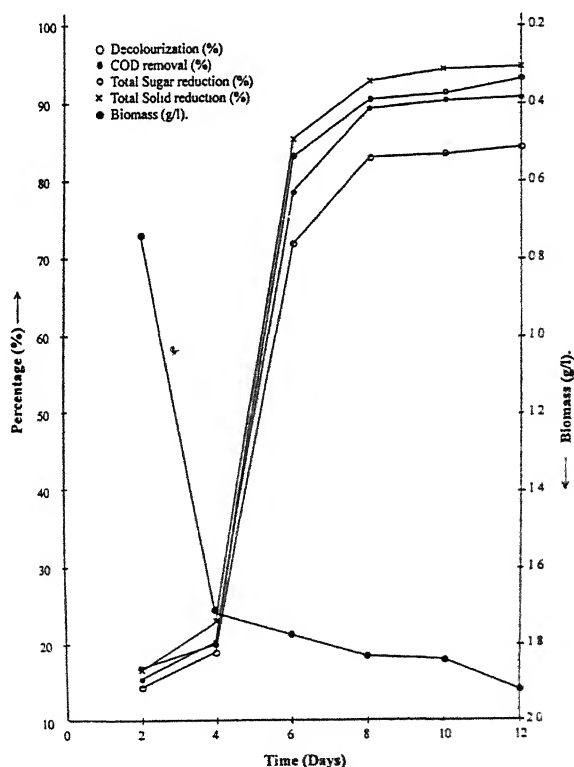


Fig. 3—Time Course of decolourization activity at optimum performance parameters.

Discussion

The present study is an attempt to improve the process of bioremediating the anaerobically digested spent wash effluent, employing an isolated fungal strain *Phanerochaete chrysosporium* GPS-V at concentration of effluent 40% (w/v), in basal medium. The fungal strain, required 6 to 8 days of incubation in attaining the maximal activity of decolourization and COD reduction at optimal performance parameters. It represents optimum growth conditions for the fungal culture which may possibly reflect the optimum activity of enzymes responsible for carrying out the decolourization. Supplementation of the basal media by readily assimilable carbon source was necessary to initiate the growth of fungus. Depletion of the supplemented carbon source in early stages of incubation may drift to refractile carbon of spent wash for further growth. This correlates the fact that the process of decolourization and growth of fungus to a certain extent depends on the uptake of the non-readily available carbon source of the effluent. In addition, glucose supplementation with various organic and inorganic sources shows a marked change on the microbial activity of decolourization. To maintain the optimum fungal nutritional conditions for the maximal activity a definite C:N ratio was maintained by supplementing 0.3% (w/v) NH_4NO_3 to basal media.

The time course data on the microbial activity of decolourization conforms to the classical scheme of Gaden¹⁶ i.e. process of decolourization considered as type II fermentation. The process was characterised by the two rate maxima with regard to growth of the culture and decolourization of the spent wash. The first phase represents the growth of the fungus accompanied by high substrate consumption i.e. readily available carbon and complex carbon of spent wash, with partial decolourization of the effluent. This may be due to the degradation of the smaller molecular weight fractions of melanoidin. The second rate maxima represents the increased rate of decolourization and reduced growth rate of fungal culture. This may be possible by the degradation of the large molecular weight melanoidins in addition to low molecular weight melanoidins by the matured fungal mycelia⁸ correlates to the rapid uptake of the sugar and reduction in the solid contents of the digested spent wash. These observations establish that the process of decolourization may occur as a result of the secondary metabolic reactions resulting from secondary metabolites. Whether it is due to sugar oxidase as speculated by Aoshima *et al.*, remains to be confirmed¹⁷.

The values of percentage decolourization, COD reduction, total solid reduction and total sugar reduction in the process of treatment of digested spent wash in the

present study are markedly higher than those reported in literature. Further, in our experiments we were able to decolourize upto 81% of the colour of 40% (v/v) digested spent wash in basal medium, which is remarkably higher as compared to the treatment of 12-24% (v/v) digested spent wash in basal medium as reported by earlier workers^{3,6,10,12,13}.

Thus an attempt of microbial treatment of digested spent wash reported here appears to be quite promising and can be developed into an industrial process.

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Intergeneric hybridization between *Atylosia* and *Cajanus*

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Abstract

Intergeneric hybrids were made between *Cajanus cajan* and three species of *Atylosia* as a female parent. Only 0.6% success could be obtained in the cross between *A. scarabaeides* \times *C. cajan*, 0.8% success was obtained in the cross *A. albicans* \times *C. cajan* and maximum 2.8% in *A. lineata* \times *C. cajan*. Important morphological characters of the three F₁ hybrids were studied at diploid level, have been compared with their respective parents. Meiotic studies revealed the nature and extent of pairing. All the hybrids were semi fertile (51%-72%). In F₂ generation, different plant types were obtained and plant types with more fodder can be used in different range and land situations.

(Key words : *Atylosia/Cajanus*/hybrids/cytology/morphology)

Introduction

Species of *Atylosia* are nutritive fodder legume, well suited in range land situations and also known as wild progenitors of *Cajanus cajan*- an important pulse crop of India. Many workers obtained hybrids between *Atylosia* and *Cajanus cajan* using *Atylosia* as male parent. They could not get success in reciprocal crosses. The presence of reciprocal differences and variation in heterosis for yield in reciprocal crosses has a great bearing in crops like pigeon pea because its uses are manifold both as human food and animal feed. the present study reports the detailed morphological and cytological analysis of successfully obtained three intergeneric hybrids and their segregants.

Materials and Methods

Seeds of materials used were obtained from the Genetic Resources Unit, ICRI SAT Hyderabad and the studies were conducted at IGFR Jhansi. Material

includes *C. cajan* (SNT coll.), *Atylosia albicans* W&A Benth (JM 2337), *A. lineata* (JM 2639), *A. scarabaeoides* (RJW coll), *A. volubilis* (JM 1984), *A. Mollis* (JM 2943), *A. platycarpa* (JM 2873). Techniques of hybridization followed was hand emasculation followed by immediate pollination. All the hybrids were identified on the morphological basis and compared with that of corresponding parents raised in the similar conditions of field and environment. For meiotic studies buds were fixed in propionoalcohol and stained in propiono carmine.

Results

Crosses were attempted in both the directions but success could be obtained only when *Cajanus* was used as a pollen parent. Percent success of crossability was 0.6, 0.8 and 2.7 in *A. scarabaeoides*, *A. albicans* and *A. lineata* respectively. Crosses of *Cajanus* with other *Atylosia* species (*A. volubilis*, *A. platycarpa* and *A. mollis*) were also attempted but success could not be obtained with these species. In these crosses only empty pods having rudimentary seeds were collected which could not germinate.

The hybrid plants exhibited dominant recessive relationship for some qualitative character, vigour for some quantitative characters and intermediate expressions for others (Table 1). In the F_1 hybrid of *A. albicans* \times *C. cajan*, leaves similar to *Cajanus cajan*, *A. albicans* and intermediate in shape were recorded on the single branch of F_1 hybrid. Pod set percent, ovule fertility and seeds per pod were lower in all the three F_1 intergeneric hybrids, when compared with their parents. In F_2 generation many plants were selected having high fodder values, good seed setting and different growth habits.

During cytological analysis of *C. cajan*, all three species of *Atylosia* and their F_1 hybrids revealed $2n=22$ chromosomes (Figs. 1-4). But in the hybrids different degrees of synopsis were observed, which is reflected in Table 2. There was slight reduction in chiasmata per cell and chiasmata per bivalent in all the inter generic hybrids. In the hybrids during late diakinesis and metaphase-1, heteromorphism was frequently observed (Fig. 3). It is also evident from the Table-1 that there was an increase in the rod bivalents and decrease in the ring bivalents in the hybrids. During meiosis at metaphase-1, one quadrivalent was observed in *A. lineata* \times *C. cajan* hybrids. A range of 0-2 trivalent could be noticed only in *A. scarabaeoides* \times *C. cajan* F_1 hybrid. At metaphase-1 univalents were of frequent occurrence. At anaphase-1 and 2 (Table 3) cytological abnormalities included irregular separation of chromosomes, laggards and chromatid bridge which resulted in the formation of micronuclei. In F_2 generation reduction in univalents formation was the prominent cytological feature. In these intergeneric hybrids pollen fertility ranged from 62.8% (*A. albicans* \times *C. cajan*) to 77.8% (*A. lineata* \times *C. cajan*).

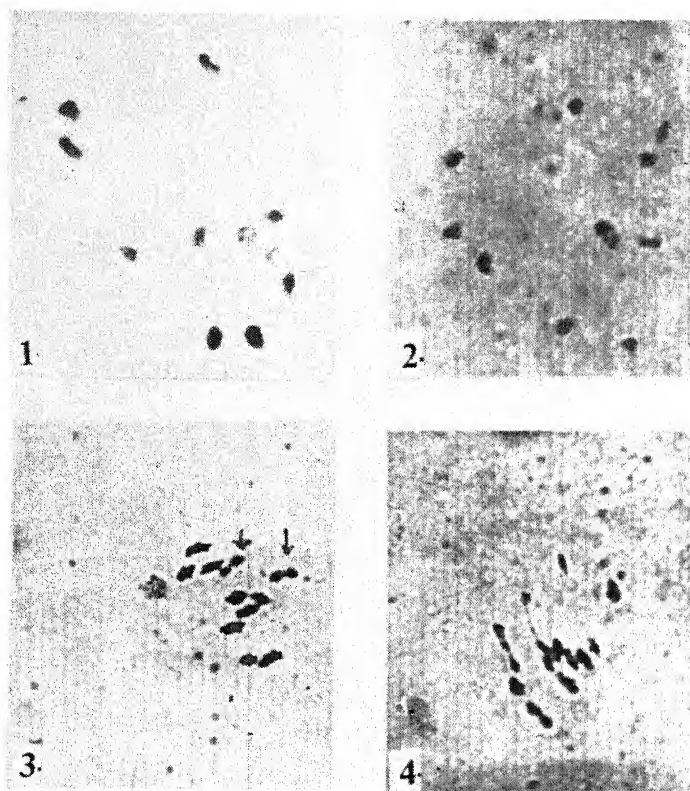


Fig. 1— 11 IIs of *Cajanus cajan* at metaphase-I (x 1500).

Fig. 2— 11 IIs of *Atylosia lineata* at metaphase-I (x 1500).

Fig. 3— 11 IIs of *A. lineata* and *Cajanus cajan* F₁ hybrids at metaphase-I showing two heteromorphic bivalents (x 1500).

Fig. 4— 10 IIs + 2Is at metaphase-I *A. lineata* x *C. cajan* F₁ hybrid (x 1500).

Table 1—Morphology of *Cajanus*, *Atylosia* spp. and their *F₁* hybrids.

Characters	<i>C. Cajan</i> (c)	<i>A. albicans</i>	<i>A. albicans</i> x (c)	<i>A. lineata</i>	<i>A. lineata</i> x(c)	<i>A. scarab.</i>	<i>A. scarab.</i> x (c)
1 Shape of first pair of leaves	Lanceolate	ovate	lanceolate	ovate	lanceolate	ovate	lanceolate
2 Growth habit	Erect shrub	twining shrub	semi erect	erect shrub	erect shrub	herbaceous creeper	semi erect
3 Plant height/spread (cm)	103	87	81	96	106	40	65.2
4 Days to flowering	105	134	124	124	134	100	123
5 Central leaflet							
Shape	Oval oblong	obovate	intermediate	lanceolate	lanceolate	obovate	intermediate
Surface	Non hairy	non hairy	non hairy	hairy	hairy	hairy	hairy
Length x breadth (cm)	4.6 x 2.6	4.2 x 3.3	6.5 x 4.3	5.2 x 2.3	5.4 x 2.3	2.5 x 1.4	3.7 x 1.5
Length of petiole (cm)	2.6	4	3.8	2.4	2.8	1.6	1.7
Leaf apices	Emerginate	oval	oval	acute	acute	acute	intermediate
6 Flower							
Colour of standard petal	Pale yellow	brownish yellow	brownish yellow	purplish yellow	purplish yellow	redish yellow	redish yellow
1 x B of standard petal (cm)	1.5 x 1.4	1.6 x 1.5	1.8 x 1.6	1.5 x 1.4	1.7 x 1.6	0.8 x 0.51	1.2 x 0.9
Nature of standard petal	Deciduous	persistant	persistant	persistant	persistant	persistant	persistant
7 Pod:							
Colour	Green	green	brown	green	brown	green	brown
1 x B (cm)	5.4 x 0.7	1.9 x 0.8	5.0 x 0.9	1.5 x 0.4	2.0 x 0.5	2.1 x 0.50	2.5 x 0.70
Hairiness	Absent	absent	absent	present	present	present	present
Nature of pod	Non shattering	shattering	shattering	shattering	shattering	shattering	shattering
8 Seed - colour	Brown	grey	grey	brown	brown	brown	brown
Seed thickness (cm)	0.002	0.28	0.502	0.3	0.323	0.2	0.302
Seeds/pod	2.2	2.1	1.5	1.82	0.33	2.5	0.6
Strophiole	Absent	present	present	present	present	present	present
9 Pod set %	26.8	61.5	15	64	4.42	64	10
10 Ovicule fertility %	85	72	44.8	83	32.8	89	20

Table 2.—Chiasma frequency and chromosome association at metaphase-I in *Cajani*, *Alydina* species and their hybrids.

Plant	Diakinesis		Xmata/ bivalent	Metaphase									
	Cells studied	Xmata/ cell		Cells studied	IV range	mean	III range	mean	II ring range	mean	II rod range	mean	I range
<i>C. cajan</i>	50	21.16	1.92	50	—	—	—	—	10.3	0-2	0.7	—	—
<i>A. albicans</i>	50	21.36	1.94	50	—	—	—	—	10.38	0-4	0.6	—	—
<i>A. lineata</i>	50	21.26	1.93	50	—	—	—	—	10.1	0-2	0.9	—	—
<i>A. scarabaeoides</i>	50	21.3	1.93	50	—	—	—	—	10.4	0-2	0.6	—	—
<i>A. albicans</i> x <i>C. cajan</i>	50	17.2	1.62	50	—	—	—	—	8.52	0-8	2.02	0-4	0.47
<i>A. lineata</i> x <i>C. cajan</i>	50	17.9	1.79	50	0-1	0.03	—	—	8.8	0-6	2.34	0-2	0.72
<i>A. scarabaeoides</i> x <i>C. cajan</i>	50	19.34	1.93	50	0-1	0.027	0-2	0.09	0.09	0-5	0.89	0-4	1.27

Table 3—Pollen fertility and chromosome separation at anaphase 1 & 2.

Plant	Anaphase-1				Anaphase-2				Pollen			Size	
	Cells studied	Normal sep. %	Laggards %	Bridge %	Cells studied	Normal sep. %	Laggards %	Bridge %	Fertility %	Bridge %	Fertility %	Range um	Mean um
<i>C. cajan</i> (c)	82	100	—	—	92	100	—	—	99.2	—	99.2	36-45	42
<i>A. albicans</i>	180	100	—	—	135	100	—	—	99.1	—	99.1	33-39	36
<i>A. lineata</i>	75	100	—	—	60	100	—	—	99.5	—	99.5	36-42	39
<i>A. scarab.</i>	120	100	—	—	200	100	—	—	99.4	—	99.4	30-35	31.5
<i>A. albicans</i> x (c)	70	95.1	2.85	1.42	80	95	3.75	1.25	62.8	—	62.8	33-45	40.5
<i>A. lineata</i> x (c)	60	94.6	3.2	1.66	100	98	2	—	77.8	—	77.8	33-45	43.5
<i>A. scarab.</i> x (c)	60	91.3	3.2	4.93	70	15.14	4.2	—	75.5	—	75.5	30-45	36.6

Discussion

The successful production of hybrids between *Atylosia* and *Cajanus* has shown that the genetic constitution of these genera/ species can produce mature plants. In the present study no success was obtained using *Cajanus cajan* as a female parent. Failure in intergeneric crosses, using, *C. cajan* as a female parent can probably be attributed to the fact that the gene mutation and selection pressure under domestication underlying the evolution of the cultivated taxon have resulted in the accumulation of the modifiers and differentiation of plasmon in the cultivated taxon *C. cajan*. These changes render *C. cajan* as an unsuccessful seed parent when cross pollinated with *Atylosia* species.

In the crosses of *C. cajan* with *A. platycarpa*, *A. mollis* and *A. volubilis* seedless pods were obtained. Cross failure may exist at the gametic, zygotic or post zygotic levels including hybrid sterility and weakness¹. In the present study it has been observed that hybrid inviability in these crosses was an active barrier at post fertilization stages, which originated from physiological incompatibility between embryo, endosperm and maternal tissue. This reaction at early stage leads to abortion of young hybrid embryo and at later stage resulted in the formation of partially filled hybrid unviable or rudimentary seeds. Similar conditions *i. e.* pods having partially filled seeds was also reported in the cross between *Phaseolus* species².

The F₁ hybrid plants exhibited dominant recessive relationship for some qualitative characters, vigour for some quantitative characters-such as plant height, branching and leaf size and intermediate expressions for others. In case of leaf size, all the F₁ hybrids exceeded to both the parents, as was reported in *C. cajan* x *A. albicnas* cross^{3,4}. In F₂ generation, on a single branch, leaves with different shapes viz, oval oblong, obovate and intermediate shapes were seen in some of the F₂ plants. Variation in leaf morphology accompanied by flowering is a consequence of differential gene expression in different branches and it is likely that this process is temporal event in gene expression. Somatic variation in *C. cajan* was reported to be chimeral which appeared from seedling stage⁵. These workers observed that somatic variation could be mutational in origin but mutation have a low probability of occurrence and are not expected to appear simultaneously in several cells of a tissue but the same is not true of treptions which may occur in all or several cells of a tissue or regions of the body, treptions are the result of a natural stimulus which triggers some regulatory process. In a previous study, in F₂ generation different plant types with more leafiness were recorded⁷.

Chromosome behavior at meiosis determines the potentiality of recombinations and it depends upon the number of chiasmata per cell and the position and distribution of chiasmata per bivalent. The factor determining these characteristics are under genetic control and are also related to chromosome size. High chiasma frequency was recorded in the intergeneric hybrids of *Atylosia* species and *Cajanus cajan*. These observations reflect that differentiation in the parental species is primarily at the genetic level which could only be maintained by geographical isolating barriers. However the possibility is there, that these species of *Atylosia* and *C. cajan* may be harbouring cryptic structural differences in their chromosome complement.

Formation of univalents can be attributed to the precocious separation of chromosomes at metaphase-1. This suggests that even the chromosomes of the two parents were apparently similar and non homology seems to exist as revealed by the formation of 0-4 univalents and heteromorphic bivalents at metaphase of these hybrids (Figs. 1-4). Formation of two or three heteromorphic bivalents was reported to be due to possible duplicated segments of chromosomes⁷ and such duplication of chromosome segment has been regarded to give opportunities for the differentiation of genus with new function and establishment of lateral heterozygosity⁸. The fact that different chromosomes of *Cajanus* exhibited heteromorphism in different hybrids of *Atylosia* suggests that these species have followed different evolutionary path way for a considerable period.

There is possibilities of selection of different plant types having more fodder potentialities with good seed setting.

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Vol. 71, Part I, 2001

CONTENTS

Review Article

- Molecular mining of the bubaline *Bubalus bubalis* genome through repetitive DNA
Sher Ali, Supriya Gangadharan Munmun Chattopadhyay, Suminder Kaur, Md. Asim Azfer and Mary Joseph Mattapallil ... 1

Animal Sciences

- Surface ultrastructure of the male external genitalia in the rock-honeybee, *Apis dorsata* F. (Hymenoptera : Apidae)
G.N. Paliwal and D.B. Tembhare ... 15
- Sperm abnormality induction assay of the drug diclofenac sodium
M. Krishnamoorthy and Subashree Laheri ... 21
- Re-examination of the microvasculature of the pseudobranch of *Mastacembelus armatus* (Lac.) and *Channa punctatus*
S. Prakash and C.B.L. Srivastava ... 27

Plant Sciences

- Bacterial Population and Diversity on "Fiddler Crab" (*Uca* sp.) at Bhavnagar Coast
A.K. Vala, S.Y. Vaidya and H.C. Dube ... 33
- Relative absorbency of some trace elements by various forms of macrophytes in a tropical water body
S. Jagdish and A. Wanganeo ... 41
- Impact of trace metals on the primary productivity of brackishwater ecosystem of Sundarbans, West Bengal
Shyamalendu Bikash Saha, S.B. Bhattacharyya and Amalesh Choudhury ... 55
- Effect of culture filtrates of some seed borne fungi on seed germination and seedling growth of Sorghum
P. Hareesh Vardhan Rao, P. Giridhar and S.M. Reddy ... 63
- Effect of root-knot nematode, *Meloidogyne incognita* on essential oil contents of rose
S.A. Tiyyagi, N. Verma and M.M. Alam ... 67
- Analysis of natural vegetation of an abandoned open cast coal mine in monsoon climate of Eastern India
Bandita Deo, P.C. Panda and P. Das ... 73

Vol. 71, Part II, 2001

Review Article	
Ion transporting enzymes and their regulation by endogenous modulators	<i>Parimal C. Sen</i> ... 83
Agricultural Sciences	
Hybridization studies between different cultivars and wild species of <i>Solanum</i> (egg plant)	<i>S.B. Agrawal, Bishwajeet Kumar and B.R. Chaudhary</i> ... 103
Animal Sciences	
Comparative analysis on food, energy and nitrogen intake of a megachiropteran bat, <i>Cynopterus sphinx</i>	<i>V. Elangovan and G. Marimuthu</i> ... 115
Laboratory rearing of <i>Cretonotus gangis</i> (Lepidoptera : Noctuidae) on artificial diet	<i>K. C. Chenchiah and A. K. Bhattacharya</i> ... 121
Environmental Sciences	
Efficacy of distillery effluent on seed germination and seedling growth in mustard, cauliflower and radish	<i>S. Ramana, A.K. Biswas, S. Kundu, J.K. Saha and R.B.R. Yadava</i> ... 129
Inter relationship amongst various physico-chemical factors, phyto and zooplankton of a lotic water body of Indo-Nepal Himalayan Terai region of Bihar	<i>Ratnesh K. Anand, Bijay Bhushan Prasad and R.B. Singh</i> ... 137
Plant Sciences	
Effect of herbal antifungal agents on 33 <i>Trichophyton</i> isolates	<i>Shweta P. Dave and H.C. Dube</i> ... 149
Effect of soil inoculation with <i>Glomus mosseae</i> at different P levels on flowering response of chrysanthemum	<i>K.B. Mamatha and D.J. Bagyaraj</i> ... 157
Production of cellulases by three isolates of <i>Syncephalastrum racemosum</i> (Cohn.) Schroet in relation to pathogenicity	<i>K. Jagadish Babu and S.M. Reddy</i> ... 165
Short Note	
A typical yield response of fibre crops to fertilizer use under rice necrosis mosaic virus inoculation	<i>Subrata Kumar Ghosh</i> ... 173

Author Index

Agrawal, S.B.	103	Kumar, Bishwajeet	103
Alam, M.M.	67	Kundu, S.	129
Ali, Sher	1	Laheri, Subashree	21
Anand, Ratnesh K.	137	Mamatha, K.B.	157
Ananthan, R.	239	Marimuthu, G.	115
Aneja, K.R.	245	Mattapallil, Mary Joseph	1
Aravinthan, K.M.	239	Morrison, M.N.	207
Ashwath, S.K.	207	Narmathabai, V.	239
Azfer, Md. Asim	1	Paliwal, G.N.	15
Babu, K. Jagadish	165	Panda, P.C.	73
Bagyaraj, D.J.	157	Panda, S.K.	255
Beena, K.R.	179	Pandey, A.K.	259
Bhattacharya, A.K.	121	Pandey, Piyush	259
Bhattacharyya, S.B.	55	Prakash, S.	27
Biswas, A.K.	129	Prasad, Bijay Bhushan	137
Chattopadhyay, Munmun	1	Prasad, B.	275
Chaudhary, B.R.	103	Ramana, S.	129
Chenchaiah, K.C.	121	Rao, P. Hareesh Vardhan	63
Choudhury, Amalesh	55	Reddy, S.M.	63,165
Das, P.	73	Saha, J.K.	129
Datta, R.K.	207	Saha, Shyamalendu Bikash	55
Dave, Shweta P.	149	Sen, Parimal C.	83
Deo, Bandita	73	Sen, Rita	223
Dolui, A.K.	233	Sharma, Chandra B.	259
Dube, H.C.	33,149	Sharma, N.C.	259
Elangovan, V.	115	Singh, N. Ibohal	275
Gangadharan, Supriya	1	Singh, R.B.	137
Ghosh, Sibdas	223	Sridhar, K.R.	179
Ghosh, Subrata Kumar	173	Srivastava, C.B.L.	27
Giridhar, P.	63	Srivastava, Kalpana	269
Gogoi, Inee	233	Tembhare, D.B.	15
Gupta, Anuja	245	Tiyagi, S.A.	67
Gupta, Sanjay	259	Tripathi, S.N.	269
Handique, Ruma	233	Vaidya, S.Y.	33
Hassarajani, S.A.	229	Vala, A.K.	33
Jagdish, S.	41	Venkatachalam, S.R.	229
Kaur, Suminder	1	Verma, N.	67
Kokate, S.D.	229	Wanganeo, A.	41
Krishnamoorthy, M.	21	Yadava, R.B.R.	129

Subject Index

Aedeagus	15	DNA probes	1
Agricultural Sciences	103	Decolourization	259
Amylase isozyme	207	Dermatophytes	149
Anaphase	223	Diclofenac sodium	21
Animal Sciences	15,21,27,115,121 223,229,233	Digested spent wash	259
Animal & Plant Cells	223	Distillery effluent	129,259
Antheraea proylei	275	Dune disturbance	179
Antifungal compounds	149	Dune vegetation	179
Apis dorsata	15	Edaphic features	179
Arbuscular mycorrhizae	179	Endophytes	179
Artificial diet	121	Energy and nitrogen requirement	115
Asymbiotic seed germination	239	Environmental Sciences	129,137
Atylosia	269	Essential oil contents	67
Backcross breeding	207	Extraction	229
Bacterial population	33	Fatty acids	229
Bioassay	229	Fertilizer	173
Biotic factors	137	Fibre crop	173
Bio-efficacy	233	Fiddler crab	33
Bubaline genome	1	Flora	73
Cadmium	255	Food choice	115
Cajanus	269	Food consumption	275
Callosobruchus chinensis	233	Foraging behaviour	115
Carbon	165	Fruit bats	115
Carotid labyrinth	27	Geographical diversity	179
Cationic amphiphilic drugs	114	Germination	63,129
Cauliflower	129	Glomus mosseae	157
Cellulases	165	Growth	63
Channa punctatus	27	Growth promotion	173
Chrysanthemum	157	Herbal medicines	149
Clerodendrum siphonanthus	233	Heterosis	103
Coal mine overburdens	73	Himalayan terai	137
Coelogyne breviscapa	239	Hybridization	103
Community analysis	73	Hybrids	103,269
Correlation coefficient	137	Impact	55
Cretonotus gangis	121	Inoculum level	67
Crossability index	103	Insecticide	233
Crustacean	33	Lipid peroxidation	255
Culture filtrate	63	Macrophytes	41
Cytology	269	Male external genitalia	15
C.nervosa	239	Mastacembelus armatus	27
C.sphinx	115	Meloidogyne incognita	67
		Metaphase	223

Modulator proteins	83	Sand aggregation	179
Molecular mining	1	Seed borne fungi	63
Morphology	269	Seed mycoflora	245
Mustard	129	Seed treatment	245
Mutagenic	21	Seed viability	245
Na ⁺ , K ⁺ -, Ca ⁺ , Mg ²⁺ - ,		Sequence evolution	1
Ca ²⁺ - ATPase regulation	83	Short Note	173,275
Near Isogenic Lines	207	Shunt vessels	27
Nitrogen sources	165	Silkworm	207
Nutrition	121	Sirkrahna river	137
Odonestis bheroba	275	Solanum	103
P fertilizer	157	Sorghum	63
Physico-chemical factors	137	Soybean	245
Plant Sciences 41,33,55,63,67,73,		Species richness	179
149,157,165,239,245,		Sperm anomaly	21
255,259,269,		Storage	245
Premature mitosis	223	Succession	179
Primary production	55	Syncephalastrum racemosum	165
Proline	255	Toxicity	229
Pseudobranch	27	Trace elements	41
P. chrysosporium	259	Trace metals	55
Quercus serrata	275	Trichophyton	149
Radish	129	Triticum aestivum	255
Rearing	121	Utilisation	275
Review Article 1,83,179,207		VA mycorrhiza	157
Rhizosphere	179	Variable loci	1
Rice necrosis mosaic virus	173	Vegetation	73
Rose	67	Zanthoxylum alatum	229

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